MANUAL OF BACTERIOLOGY

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MUIR & RITCHIE'S
MANUAL OF BACTERIOLOGY

REVISED BY

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TENTH EDITION

WITH 212 ILLUSTRATIONS IN THE TEXT AND 6 COLOURED PLATES

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PREFACE TO THE TENTH EDITION

SINCE the issue of the last edition there have been again numerous advances in practically every department of Bacteriology. In particular, knowledge regarding filterable viruses has been much extended. Not only has the number of diseases in which viruses are the causal agents been largely added to; but our understanding of those previously studied has become much fuller, and this is due in great measure to the introduction of novel methods of investigation. The importance of bacterial variation has been increasingly recognised. The close interrelationships of infections in animals and man have received fresh emphasis from newer work. Also, the examination of the constitution of antigens by chemical as well as serological methods has furnished abundant data which promise to elucidate further the behaviour of many of the pathogenic bacteria. The incorporation of these and many other recent advances has necessitated extensive revision of every chapter, so that there is scarcely any section which has not been rewritten in great part. Some increase in size has therefore been inevitable. In dealing with the important pathogenic organisms the old-established names have been retained while those of the new classification have been added as alternatives. This has been done advisedly, since the classification of bacteria is still so unsettled that a satisfactory nomenclature on such a basis is not yet feasible.

Owing to the withdrawal of Sir Robert Muir from active

participation in the revision, the original authorship has now come to an end. But it has been the aim of the present writers throughout to continue the work in the spirit which has inspired it in the past and which has commended itself so widely.

The illustrations have been extensively replaced, and the drawings and most of the new photo-micrographs are by Mr. John Kirkpatrick of the Pathology Department, The University, Glasgow; some of the latter are by Mr. Alex. Cheyne of the Bacteriology Department, The University, Edinburgh.

We are indebted for information on special subjects to Professor C. P. Beattie, Lieut-Col. E. D. W. Greig, Dr. W. B. Kyles, Dr. G. H. Percival, Dr. C. E. van Rooyen, and Dr. Malcolm Wilson.

C. H. B.
T. J. M.

October 1937.

CONTENTS

CHAPTER I

PAGE

General Morphology and Physiology; Bacterial Variation; Bacteriophage 1

CHAPTER II

Methods of Cultivation of Bacteria . . . 49

CHAPTER III

Microscopic Methods 106

CHAPTER IV

Serological Methods—Preparation of Vaccines—Inoculation of Animals—Methods of obtaining Pathological Material for Examination . . . 136

CHAPTER V

Relations of Bacteria to Disease—Infection—Production of Toxins by Bacteria 171

CHAPTER VI

Immunity and Supersensitiveness 200

CHAPTER VII

Pyogenic Cocci and Other Bacteria associated with Inflammatory and Suppurative Conditions: Staphylococci; Streptococci; Organisms of Conjunctivitis; Micrococcus Tetragenus; Bacillus Pyocyaneus; Bacillus Proteus; Ducrey's Bacillus . 274

CHAPTER VIII

PAGE

Bacteria Associated with Inflammatory and Suppurative Conditions (*continued*) : The Pneumococcus and Pneumobacillus ; The Meningococcus and Gonococcus 325

CHAPTER IX

The Diphtheria Bacillus and Allied Organisms ; Bacterium Monocytogenes 373

CHAPTER X

The Tubercle Bacillus ; Non-pathogenic Acid-fast Bacilli ; Bacillus of Johne's Disease . . . 405

CHAPTER XI

The Leprosy Bacillus 447

CHAPTER XII

The Bacilli of Glanders and Melioidosis . . . 456

CHAPTER XIII

Actinomyces (Streptothrix Group) and Allied Organisms ; Bacillus Necrophorus ; Leptothrix ; Erysipelothrix ; Organisms of Bovine Pleuropneumonia and Agalactia 466

CHAPTER XIV

The Anthrax Bacillus and Allied Organisms . . . 486

CHAPTER XV

The Coli-Typhoid Group and Allied Organisms Bacillus Coli, Bacillus Typhosus, Bacillus Paratyphosus and Organisms of Food-poisoning (Salmonella Group) ; Dysentery Bacilli ; etc. 506

CHAPTER XVI

Vibrio Choleræ and Allied Organisms 560

CHAPTER XVII

PAGE

Bacillus Influenzæ and Allied Organisms; Bacillus Pertussis; Bacterium Pneumosintes; Streptobacillus Moniliformis 579

CHAPTER XVIII

Bacillus Pestis and Allied Organisms (Pasteurella Group); Bacterium Tularense 596

CHAPTER XIX

The Organisms of Undulant Fever and Epizootic Abortion (Brucella Group) 615

CHAPTER XX

The Tetanus Bacillus and other Pathogenic Anaerobic Bacilli; Bacillus Fusiformis 625

CHAPTER XXI

Pathogenic Spirochætes: Treponema Pallidum and Treponema Pertenue 666

CHAPTER XXII

Spirochætes of the Relapsing Fevers; Leptospira Icterohæmorrhagiæ and Allied Organisms; Spirillum Minus 682

CHAPTER XXIII

Rickettsia Group (of Typhus Fever, Trench Fever, etc.) 703

CHAPTER XXIV

Filterable Viruses; Smallpox and Vaccinia; Rabies . 715

CHAPTER XXV

Filterable Viruses (*continued*); Poliomyelitis; Encephalitis; Herpes; Chicken-pox; Measles; Mumps; Influenza; Common Cold; Psittacosis; Yellow Fever; Dengue; Rift Valley Fever; Phlebotomus Fever; Climatic Bubo; etc. 750

CHAPTER XXVI

PAGE

PATHOGENIC PROTOZOA ; MALARIA PLASMODIA ; ENTAMŒBA HISTOLYTICA (OF TROPICAL DYSENTERY) . . . 789

CHAPTER XXVII

PATHOGENIC PROTOZOA (*continued*) : TRYPANOSOMES , LEISHMANIÆ ; PIROPLASMS ; BARTONELLA . . . 823

CHAPTER XXVIII

PATHOGENIC FUNGI 858

CHAPTER XXIX

BACTERIOLOGY OF AIR, SOIL, WATER, SEWAGE, MILK ; ANTISEPTICS ; CHEMOTHERAPEUTIC AGENTS . . . 885

APPENDIX

THE BACTERIAL FLORA OF THE NORMAL SKIN AND MUCOUS MEMBRANES 920

BIBLIOGRAPHY 925

INDEX 969

PLATE I.

Fig. 1. Film of pus, containing staphylococci and streptococci. Stained by Gram's method. × 1000 diameters.

Fig. 2. Pneumococci in sputum, from a case of acute pneumonia. Rd. Muir's method of capsule staining. × 1000 diameters.

Fig. 3. Meningococci in epidemic cerebro-spinal fever, from lumbar-puncture fluid, showing some involution forms. Leishman's stain. × 1000 diameters.

Fig. 4. Film from a scraping of throat in Vincent's angina, showing fusiform bacilli and spirochætes. Leishman's stain. × 1000 diameters.

Fig. 5. Gonorrhœal pus, showing gonococci (stained red) and staphylococci. Gram's method. × 1000 diameters.

PLATE I.

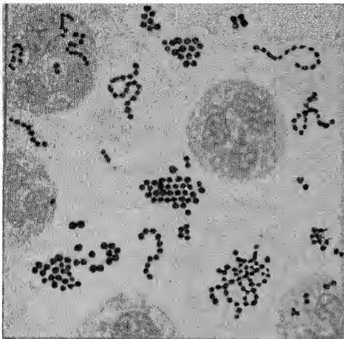
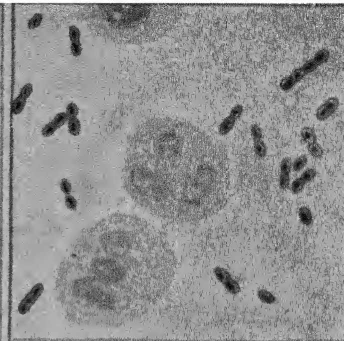


Fig. 1.



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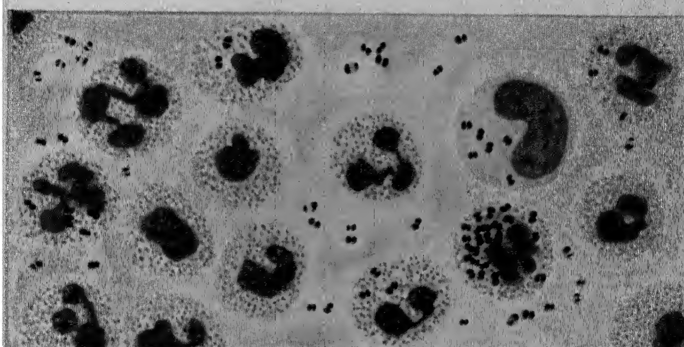

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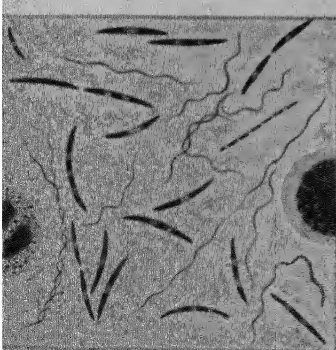

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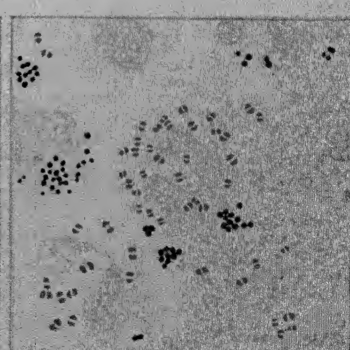

Fig. 5.

PLATE II.

FIG. 6. Treponema pallidum in section of spleen of child; case of congenital syphilis. Levaditi's stain. × 1000 diameters.

FIG. 7. Tubercle bacilli and other bacteria in sputum; case of pulmonary phthisis. Ziehl-Neelsen stain. × 1000 diameters.

FIG. 8. Section of leprous skin, showing numerous clumps of bacilli (stained red) in the cutis. Carbol-fuchsin and methylene-blue. × 80 diameters.

FIG. 9. Section of leprous granulation tissue, showing large numbers of bacilli, chiefly contained within cells. Carbol-fuchsin and methylene-blue. × 1000 diameters.

PLATE II.

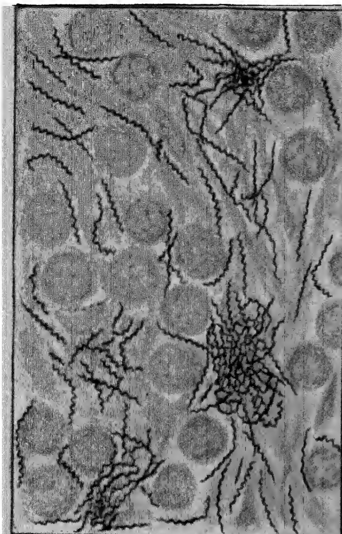

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FIG. 7.

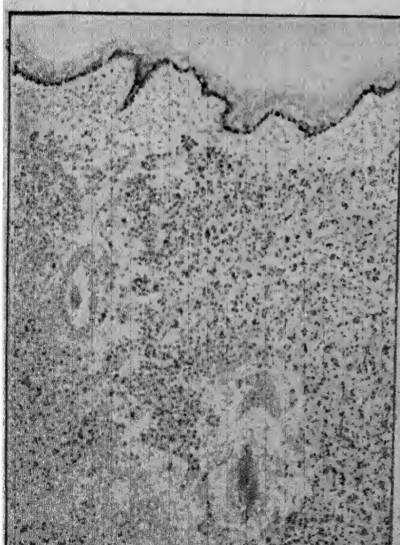


FIG. 8.

FIG. 9.

PLATE III.

FIG. 10. Streptothrix actinomyces from agar culture. Gram's method. × 1000 diameters.

FIG. 11. Anthrax bacilli, from a 4-days' agar culture showing spores. Carbol-fuchsin and methylene-blue. × 1000 diameters.

FIG. 12. Bacillus diphtheriæ, from an 18-hours' culture on Löffler's serum. Neisser's method. × 1000 diameters.

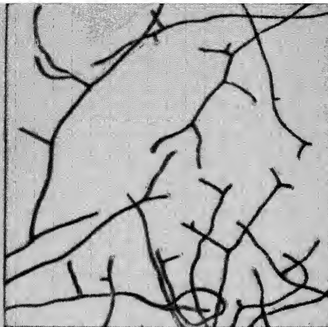
FIG. 13. Bacillus diphtheriæ, from an 18-hours' culture on Löffler's serum. Methylene-blue. × 1000 diameters.

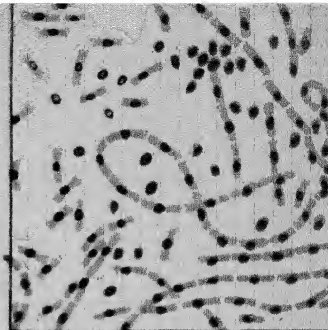
FIG. 14. Bacillus diphtheriæ, from a 5-days' culture on Löffler's serum, showing involution forms. Methylene-blue. × 1000 diameters.

FIG. 15. Pseudo-diphtheria bacillus (Hofmann's), from an 18-hours' culture on Löffler's serum. (*a*) Neisser's method; (*b*) Methylene-blue.
To compare with Figs. 12, 13. × 1000 diameters.

FIG. 16. Typhoid bacilli from a 24-hours' agar culture, showing flagella. Rd. Muir's method. × 1000 diameters.

PLATE III.





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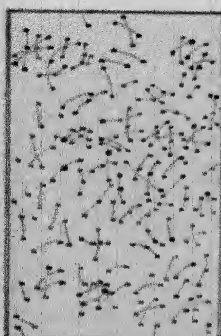

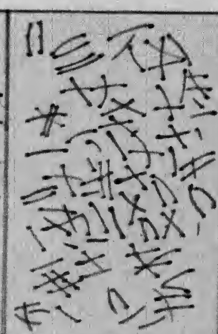

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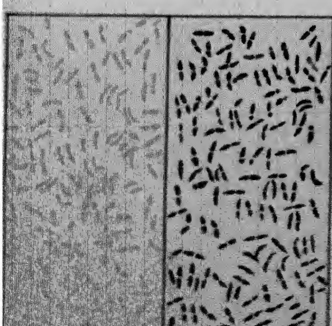


a FIG. 15. *b*

FIG. 16.

PLATE IV.

Fig. 17. Negri bodies in nerve cells in rabies (hippocampus of dog). Alcoholic eosin and methylene-blue. × 1000 diameters.

Fig. 18. Bacillus pestis, showing involution forms, from a salt-agar culture. Thionin. × 1000 diameters.

Fig. 19. Blood film, showing the spirochæte of relapsing fever. Leishman's stain. × 1000 diameters.

Fig. 20. Vibrio choleræ, from a 12-hours' agar culture, showing flagella. Rd. Muir's method. × 1000 diameters.

Fig. 21. Bacillus tetani, showing spores. Dilute carbol-fuchsin. × 1000 diameters.

PLATE IV.

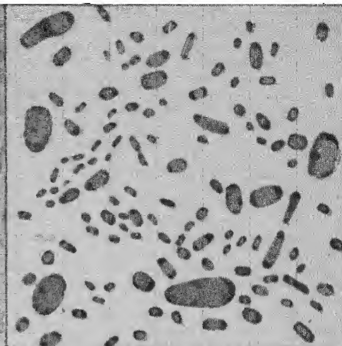
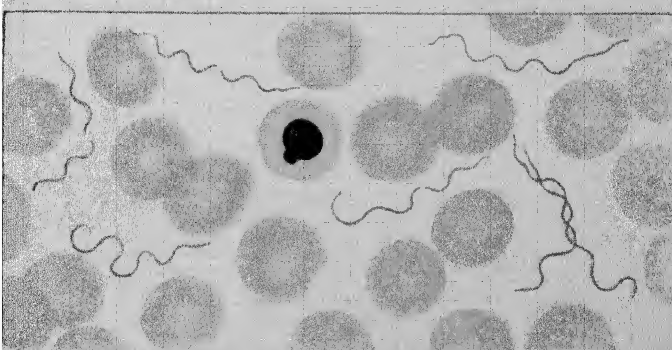


FIG. 17.

FIG. 18.



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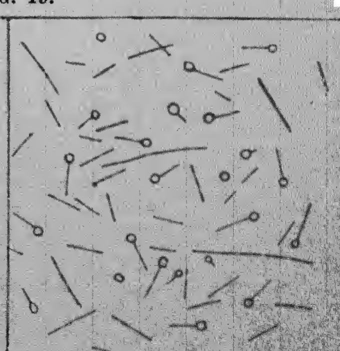


FIG. 20

FIG 21

PLATE V.

FIG. 22. THE PARASITE OF BENIGN TERTIAN MALARIA.

Cycle I. (Schizogony). Asexual cycle in human blood.

a. Sporozoite entering red blood corpuscle and forming young trophozoite.
b. Young trophozoite in red blood corpuscle.
c. Young trophozoite in red blood corpuscle with pigment appearing.
d. Large pigmented trophozoite.
e. Mature schizont.
f. Commencing segmentation of schizont—schizogony.
g. Further stage of segmentation.
h. Segmented schizont; formation of merozoites.
i. Disintegration of red blood corpuscle, setting free the merozoites.
j. Young merozoite entering red blood corpuscle.
k. Macrogametocyte (female).
l. Microgametocyte (male).

Cycle II. (Sporogony). Sexual cycle in the mosquito.

In the cavity of the stomach of the mosquito.
m. Microgametocyte.
n. Macrogametocyte.
o. Formation of microgametes from the microgametocyte.
p. Free microgamete.
q. Microgamete entering the macrogamete.
r. Zygote or ookinete.

s. Sporocyst.
t. Formation of sporoblasts in the sporocyst.
u. Formation of sporozoites from sporoblasts.
v. Rupture of sporocyst, setting free the sporozoites.
w. Free sporozoites in the body fluid.
x. Accumulation of sporozoites in the salivary gland.
y. Sporozoites passing from gland duct into the blood of man.

FIG. 23. THE PARASITES OF MALIGNANT MALARIA.

a. Young trophozoite entering red blood corpuscle.
b. Do. in red corpuscle.
c. Multiple infection of red corpuscle.
c′. Multiple infection with chromatic stippling in cellular protoplasm; a similar cell is seen lying beneath *a*,—it contains a pigmented trophozoite.
d. Pigmented trophozoite.
e. Segmented schizont, cluster of merozoites.
f. Microgametocyte, "male crescent."
g. Macrogametocyte, "female crescent."
h. Red blood corpuscle with chromatic stippling.
i. Large mononuclear phagocyte containing malarial pigment.

PLATE V.

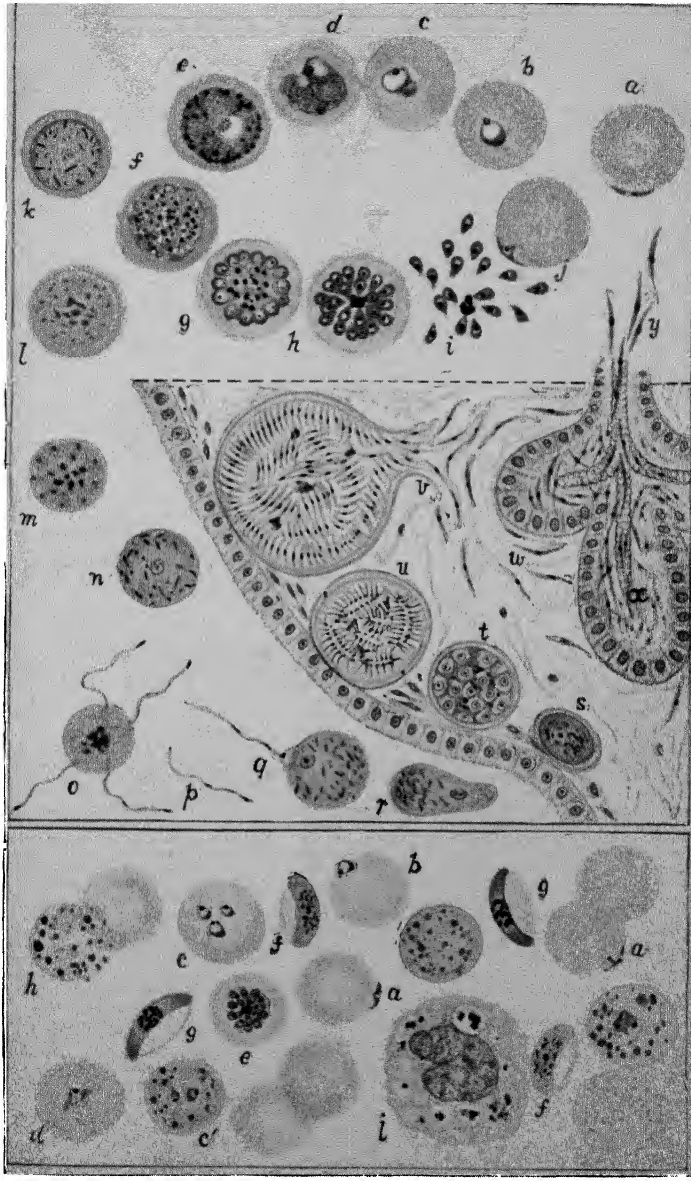


FIG. 23.

PLATE VI.

Fig. 24. Entamœba histolytica in pus, from tropical abscess of liver. Wet-fixed film. Stained with safranin and light green. × 1000 diameters.

Fig. 25. Leishman-Donovan bodies, from the spleen of a case of kala-azar. Leishman's stain. × 1000 diameters.

Fig. 26. Blood-film showing Trypanosoma gambiense. Leishman's stain. × 1000 diameters.

PLATE VI.

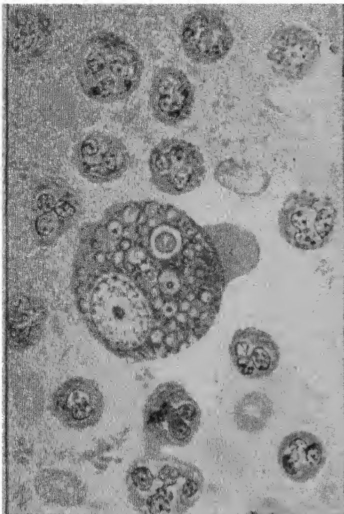


Fig. 24.

Fig. 25.

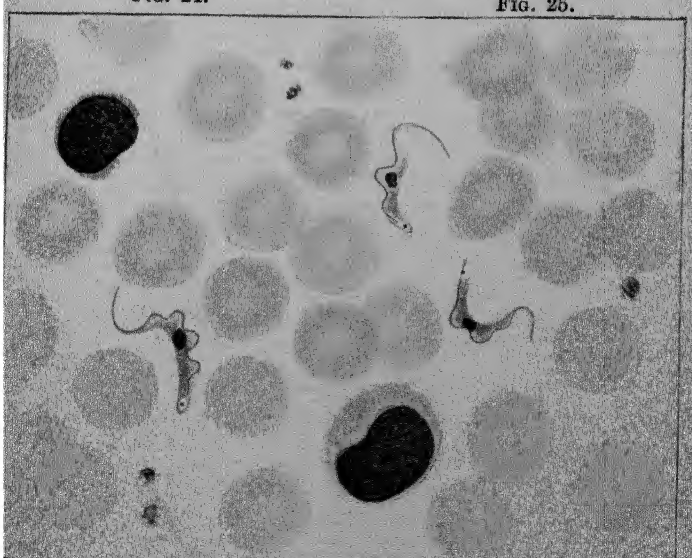


Fig. 26.

MANUAL OF BACTERIOLOGY

CHAPTER I

GENERAL MORPHOLOGY AND PHYSIOLOGY

Introductory.—Micro-organisms were first observed by Leeuwenhoek and in 1683 he figured different types of bacteria present in material from between the teeth. It was not until the work of Pasteur, however, that proof could be brought concerning the causal relation of such minute forms of life to the processes of fermentation and putrefaction and of infective diseases. Pasteur demonstrated conclusively by simple experiments that spontaneous generation of living micro-organisms does not occur. Accordingly it follows that their presence in any material is due to their introduction in the living state and subsequent multiplication under suitable conditions. The micro-organisms which are responsible for the infective diseases of man and animals include primitive forms, both of the plant and animal kingdoms. The causal agents of the majority of infective conditions in countries with temperate climates are bacteria, which are accepted as being among the lowest forms of plant life; and with these we are chiefly concerned. In addition, however, higher plant forms, corresponding to the moulds and yeasts, namely, true fungi, and also the lowest members of the animal kingdom, the protozoa, require consideration in view of the occurrence among them of many pathogenic species. Classification of unicellular organisms is difficult, and has been the subject of much controversy. Certain micro-organisms are also difficult to assign to either the plant or animal kingdom, and Haeckel, in order to simply matters, introduced the term "Protista" to include the lowest members of both kingdoms. General agreement amongst systematists is, however, now being attained and the principles of classification are becoming

stabilised, though there still remain matters of doubt or controversy. Further, it is now well recognised that various important and prevalent diseases are due to "viruses" that are not demonstrable by ordinary methods, owing to their size being beyond the limits of resolution by the microscope, hence called *ultramicroscopic viruses*. Such viruses can be separated from other micro-organisms by their property of filterability through an earthenware filter, and thus they are often known as *filterable viruses* or *filter-passers*. These terms have, however, only a relative significance, as some of the filterable viruses have recently been defined as very minute microscopically visible bodies, and a particle may pass through or be retained according to the character of the filter, conditions of filtration, etc.; also, some organisms which are microscopically visible may pass filters under certain circumstances. The question whether these viruses are living organisms has been a matter of controversy, and constitutes one of the most interesting and fundamental of biological problems. The more important diseases produced by the filterable viruses will also receive consideration.

The bacteria collectively form the class designated *Schizomycetes*. They are unicellular organisms of various forms, devoid of chlorophyll and with imperfectly differentiated nucleus, and multiply in most cases by simple fission; some are motile, others are non-motile. They are of comparatively simple structure and in one system of classification have been arranged in two main subdivisions—a lower and simpler, and a higher and more organised.

The *lower forms* or *Eubacteria* are the more numerous, and are minute, relatively undifferentiated masses of protoplasm, which produce similar cells by simple fission. Some are motile, others non-motile. Their minuteness may be judged by the fact that in one direction at least they usually do not measure more than 1 μ ($\frac{1}{1000}$ millimetre or $\frac{1}{25000}$ inch). These forms can be broadly classified according to their shapes into four main groups—(1) A group in which the shape is approximately globular. A member of this is called a *Coccus*. (2) A group in which the shape is that of a straight rod—the proportion of the length to the breadth of the rod varying greatly among the different members. This form is called a *Bacillus*. (3) A group in which the shape is that of a curved rod (*Vibrio*) or a non-flexuous spiral filament (*Spirillum*). (4) A group of filamentous flexuous organisms showing undulations or true spirality. An organism of this type is designated a *Spirochæte*. There has been much dispute with regard to the classification

of this group. Many of them have been regarded as protozoon-like and belonging to the animal kingdom; they are now, however, generally placed amongst the bacteria. Further, all the spirochætes have been grouped in the American classification as a distinct order of the Schizomycetes co-equal with the Eubacteria (p. 33). A detailed description of the characters of these groups will be more conveniently taken later (p. 33 *et seq.*). In some cases, especially among the bacillary forms, there may occur under certain circumstances changes in the protoplasm whereby a resting phase or spore is formed. It should be noted that while many species of bacteria are very constant in their microscopic appearances, others show marked differences depending on conditions affecting their environment and the age of the culture. Such pleomorphism, as it has been termed, may be a source of difficulty in the identification of organisms (p. 38).

The *higher bacteria* show advance on the lower along two lines. (1) On the one hand, they consist of filaments made up of simple elements such as occur in the lower forms. These filaments may be more or less septate, may be provided with a sheath, and may show branching, either true or false. The minute structure of the elements comprising these filaments is analogous to that of the lower forms. Their size, however, is often somewhat greater. The lower forms sometimes occur in filaments, but here every member of the filament is independent, while in the higher forms there seems to be a certain interdependence among the individual elements. For instance, growth may occur only at one end of a filament, the other forming an attachment to some fixed object. (2) The higher forms, moreover, present this further development, that in certain cases some of the cells may be specialised reproductive units (p. 31).

The relations of the bacteria to the animal kingdom on the one hand and to the plants on the other constitute a difficult question. It is best to think of there being a group of small, unicellular organisms, which may be survivals of the most primitive forms of life before differentiation into animal and plant types had occurred and in some cases even before, in an individual cell, nucleus had been morphologically differentiated from cytoplasm. This would include the protozoa the myxomycetes, the lower algæ, and the bacteria. To the lower algæ the bacteria show many similarities. These algæ are unicellular masses of protoplasm, having generally the same shapes as the bacteria, and largely multiplying by fission. Endogenous sporulation, however, does not occur, nor is motility necessarily associated with the possession of flagella. Also their protoplasm differs from that of the bacteria in containing chlorophyll and another blue-

green pigment called phycocyan. From the morphological resemblances between these algæ and the bacteria, and from the fact that fission plays a predominant part in the multiplication of both, they were formerly grouped together in one class as the Schizophyta or splitting plants. And of the two divisions forming these Schizophyta the splitting algæ were denominated the Schizophyceæ, while the bacteria or splitting fungi were called the Schizomycetes.

General Morphology of the Bacteria

The Structure of the Bacterial Cell.—When examined under the microscope, in their natural condition, *e.g.* in water, bacteria appear merely as colourless refractile bodies of the different shapes named. Spore formation and motility, when these exist, can also be observed, but little else can be made out. For their proper investigation advantage is taken of their affinities for various dyes, especially those which stain intensely the nuclei of animal cells. Certain points have thus been determined. The bacterial cell consists of a sharply contoured mass of protoplasm which reacts to basic aniline dyes like the nucleus of an animal cell. A healthy bacterium when thus stained commonly presents the appearance of a finely granular or almost homogeneous structure. The protoplasm is surrounded by an envelope or "ectoplasm" which can in some cases be demonstrated by overstaining the specimen, when it will appear as a halo round the bacterium. This envelope is of special importance in relation to the staining reactions of bacteria (p. 117). Its innermost layer is probably of a denser consistence, and sharply contours the contained protoplasm, giving the latter the appearance of being surrounded by a membrane. It is only, however, in some of the higher forms that a definite sheath occurs. Sometimes the outer margin of the envelope is sharply defined, in which case the bacterium appears to have a distinct capsule, and is known as a capsulated bacterium (*vide* Fig. 2, *g*; and Fig. 44). The cohesion of bacteria into masses depends largely on the character of the envelope. If the latter is glutinous, then a large number of the same species may occur, formed of individual bacteria embedded in what appears to be a matrix of jelly; this is known as a *zooglœa* mass. On the other hand, if the envelope has not this cohesive property the separation of individuals may easily take place, especially in a fluid medium in which they may float entirely free from one another. Many of the higher bacteria possess a sheath which has a much more definite structure than is found among the lower forms. It resists external influences, possesses

elasticity, and serves to bind the elements of the organism together. In certain forms the sheath contains granules of iron oxide or other substances. A number of pathogenic bacteria produce definite capsules especially when growing in the tissues, and in some cases a relationship has been established between capsule formation and virulence. In several cases carbohydrate materials have been shown to be important constituents of capsules.

Reproduction among the Lower Bacteria.—When a bacterial cell is placed in favourable surroundings, *e.g.* a suitable culture medium (*vide* Chap. II.), it multiplies by simple fission. In the process a constriction appears in the middle and a transverse unstained line develops across the protoplasm at that point. The process goes on till two individuals can be recognised, which may remain for a time attached to one another, or become separate, according to the character of the envelope, as already explained. In most bacteria growth and multiplication go on with great rapidity. A bacterium may reach maturity and divide in from twenty minutes to half an hour. If division takes place only every hour, from one individual after twenty-four hours 17,000,000 similar individuals will be produced. As shown by the results of artificial cultivation, certain bacteria, e.g. *B. tuberculosis*, multiply much more slowly. In some cases the bacterial cell enlarges before division, in others the cell divides and each element then expands to its adult size. If, in the latter case, multiplication is proceeding rapidly, great variation in the size of the individuals may be observed, and this may give rise to anomalous appearances. Among the spirochætes longitudinal as well as transverse division has been described, though this is doubtful.

In some cases division occurs in an irregular manner. Thus among the bacilli branched Y-shaped structures may develop and new individuals may be formed at each branch (" three-point multiplication "). Sometimes a constriction forms near the end of a bacillus and a spheroidal segment is separated off; such aberrant elements, however, may not develop further. Unequal division may also result among the cocci, giving rise to rod-shaped forms.

When a small number of bacteria are brought into fresh medium the immediate course of events depends on the state of the culture which furnished the inoculum. If the culture is an old one in which active multiplication of organisms has ceased, then on transference to the new medium reproduction does not start at once; instead there is a period of delay, the

"lag phase," before rapid multiplication starts. This is followed by a period of maximal growth in which the number of cells increases in geometric progression with the time. Thereafter the rate of division slows down and finally becomes negligible; ultimately the culture may die. When the inoculum is derived from a culture in the phase of maximal growth, there is no lag on transference to fresh medium. Accordingly, the lag phase may be interpreted as evidence that organisms in an old culture have passed into a condition of depressed vitality.

It should be noted here that when bacteria are sparsely distributed on the surface or in the substance of a solid medium, the resulting growth develops in the form of separate "colonies" which may be visible to the naked eye. Furthermore, such colonies may present appearances which are characteristic of bacterial groups or species (see various illustrations in later chapters).

From investigations by Graham-Smith and others, it appears that the consistence of the envelope may have an importance in modifying the naked-eye and low-power appearances presented by bacterial colonies, which constitute a feature in the identification of species. Graham-Smith has differentiated four groups — a "loop-forming," in which the envelope is so tough that, after division, rupture but rarely occurs (*B. anthracis*); a "folding" group, in which the envelope is so flexible and extensile that the members of a chain can be folded on one another as successive divisions take place (*B. pestis*); a "snapping" group, in which partial rupture of the envelope occurs on division (*B. diphtheriæ*); and a "slipping" group, where the envelope readily breaks, and successively developed bacteria slip past each other (*V. choleræ*).

When bacteria are placed in unfavourable conditions as regards food, etc., growth and multiplication take place with difficulty. In the great majority of cases this is manifested by changes in the appearances of the protoplasm. Instead of its maintaining the regularity of shape seen in normal bacteria, various aberrant appearances are presented. This occurs especially in the rod-shaped varieties, in which flask-shaped or dumb-bell-shaped individuals may be observed. The regularity in structure and size is quite lost. The appearance of the protoplasm also is often altered. Instead of, as formerly, staining well, it does not stain readily, and may have a uniformly pale homogeneous appearance, while in an old culture only a small proportion of the bacteria may stain at all. Sometimes, on the other hand, a degenerated bacterium contains intensely stained granules or globules which may be of large size. Such

aberrant and degenerate appearances are referred to as ***involution forms*** (Fig. 2, s^1-s^4). That these forms really betoken degenerative changes is shown by the fact that on their being again transferred to favourable conditions, only slight growth at first takes place. Many individuals have undoubtedly died, and the remainder which live and develop into typical forms may sometimes have lost some of the original properties of the strain.

While it is generally accepted that fission is the sole mode of reproduction among the lower bacteria, there have also been described budding and the formation of granules (gonidia) within the cells, which subsequently become free ; these granules may be surrounded by a resistant layer—arthrospores. Certain observers have also described the occurrence of life cycles (cyclogeny) including sexual reproduction by conjugation, and " symplasm " formation ; in the latter the organisms lose their individual form and fuse into masses from which later new cells arise. Irregular proliferation of the nature of budding seems to occur in certain organisms, and strong evidence of the formation of gonidia has been brought forward in the case of *B. radicicola* ; but it is doubtful whether these processes ever occur in the reproduction of the pathogenic bacteria. As regards life cycles, the views seem to depend on the interpretation attached to appearances which have been described above as involution forms ; no definite proofs of such cycles have been so far brought forward.

The reproductive processes among the higher bacteria are referred to later.

Spore Formation.—In certain species of the lower bacteria, under certain circumstances, changes take place in the protoplasm which result in the formation of bodies called spores, to which the vital activities of the original bacteria are transferred. Spore formation occurs chiefly among the bacilli. Its development in a bacterium is indicated by the appearance in the protoplasm of a highly refractile globular structure unstained by the ordinary methods. This may increase in size, and assume a round, oval, or short rod-shaped form, always shorter but often broader than the original bacterium. In the process of spore formation the rest of the bacterial protoplasm may remain unchanged in appearance and staining power for a considerable time (e.g. *B. tetani*), or, on the other hand, it may soon lose its power of staining and ultimately disappear (e.g. *B. anthracis*). This method of spore formation is called ***endogenous***, and the spores are known as ***endospores***. Bacterial spores are

always non-motile. The spore may appear in the centre of the bacterium (*central*), or it may be at one extremity (*terminal*), or a short distance from one extremity (*subterminal*) (Fig. 2, l^1-l^6). In structure the spore consists apparently of a mass of protoplasm surrounded by a dense membrane or capsule. This can be demonstrated by methods which will be described, the underlying principle of which is the prolonged application of a powerful stain. The all-important property of a bacterial spore is its high degree of resistance to external influences such as heat, drying, chemical agents, etc. Such resistance has been ascribed to the capsule and to the very small amount of water in the contents. Koch, for instance, in one series of experiments, found that while the *Bacillus anthracis* in the unspored form was killed by a two minutes' exposure to 1 per cent. carbolic acid, spores of the same organism resisted an exposure of from one to fifteen days.

When a spore is placed in suitable surroundings for the vegetative growth of the organism, it "germinates" and again assumes the original bacillary form. The capsule may dehisce either longitudinally, or terminally, or transversely. In the last case the dehiscence may be partial, and the new individual may remain for a time attached by its ends to the hinged spore-case, or the dehiscence may be complete and the bacillus grow with a cap at each end consisting of half the spore-case. Sometimes the spore-case does not dehisce, but is simply absorbed by the developing bacterium. Even under conditions suitable for vegetative growth, a proportion of spores of various species may lie dormant for several months before beginning to germinate (Burke).

It is important to note that, in the bacteria, spore formation is rarely, if ever, to be considered as a method of multiplication. In at least the great majority of cases only one spore is formed from one bacterium, and only one bacterium in the first instance from one spore. Sporulation is to be looked upon as a *resting phase* of a bacterium, and is to be contrasted with the stage when active multiplication takes place. The latter is usually referred to as the *vegetative phase* of the bacterium. Regarding the significance of spore formation in bacteria, there has been some difference of opinion. According to one view, it may be regarded as representing the highest stage in the vital activity of a bacterium. There is thus an alternation between the vegetative and spore stage, the occurrence of the latter being necessary to the maintenance of the species in its greatest vitality. Such a rejuvenescence, as it were, through sporulation,

is known in many algæ. In support of this view there are certain facts. In many cases, for instance, spore formation only occurs at temperatures specially favourable for growth and multiplication. There is often a temperature below which, while vegetative growth still takes place, sporulation will not occur; and in the case of *B. anthracis*, if the organism be kept at a temperature above the limit at which it grows best, not only are no spores formed, but the strain may lose the power of sporulation. Furthermore, in the case of bacteria preferring the presence of oxygen for their growth, an abundant supply of this gas may favour sporulation. It is probable that even among bacteria preferring the absence of oxygen for vegetative growth, the presence of oxygen favours sporulation. The second view with regard to sporulation is that a bacterium only forms a spore when its surroundings, especially its food supply, become unfavourable for vegetative growth; it then remains in this condition until it is placed in more suitable surroundings. Such an occurrence would be analogous to the *encystment* which occurs under similar conditions in many of the protozoa. Often sporulation can be prevented from taking place for an indefinite time if a bacterium is constantly supplied with fresh food (the other conditions of life being equal). The presence of substances produced by the bacteria themselves plays, however, a more important part in making the surroundings unfavourable than the mere exhaustion of the food supply. A living spore will always develop into a vegetative form if given a fresh food supply. With regard to the rapid formation of spores when the conditions are favourable for vegetative growth, it must be borne in mind that in such circumstances the conditions may actually very quickly become unfavourable for a continuance of growth, since not only will the food supply around the growing bacteria be rapidly exhausted, but the formation of inimical products will be all the more rapid.

We must note that the usually applied tests of a body developed within a bacterium being a spore depend on (1) its staining reaction, namely, resistance to ordinary staining fluids, but capacity of being stained by the special methods devised for the purpose (*vide* p. 122); (2) the fact that the bacterium containing the spore has higher powers of resistance against inimical conditions, *e.g.* heat, than a vegetative form. It is important to bear these tests in mind, as, in some of the smaller bacteria especially, it is very difficult to say whether they sporulate or not. There may appear in such organisms small unstained spots, the significance of which is very difficult to

determine; in every case the test of resistance must be made. There is a considerable difference in the resistance of spores of different species; also spores of the same species may vary to a marked degree in this respect among themselves.

Motility.—As has been stated, many bacteria are motile. Motility can be studied by means of hanging-drop preparations (*vide* p. 107). The movements are of a darting, rolling, or vibratile character. The degree of motility depends on the species, the temperature, the age of the growth, and on the medium in which the bacteria are growing. Sometimes the movement is most active just after the cell has multiplied, sometimes it goes on all through the life of the bacterium, sometimes it ceases when sporulation is about to occur. Motility is associated with the possession of fine wavy thread-like appendages called flagella, which for their demonstration require the application of special staining methods (*vide* Fig. 2, k^1-k^3 and Fig. 110). They have been shown to occur in many bacilli and spirilla, but only in a few species of cocci. They vary in length, but may be several times the length of the bacterium, and may be at one or both extremities or all round. When terminal they may occur singly or there may be several; in some spirilla a tuft of terminal flagella is present. Flagella may be regarded as spirals which are directed backwards and rotate in the opposite direction to the body of the organism, thus conferring on it a forward movement. Sometimes complicated spiral tresses of detached flagella are found in bacterial cultures. The nature of flagella had been much disputed. It is generally held that they are actual prolongations of the bacterial protoplasm. In all probability they are derived from the "ectoplasm" of the cell and studies of bacterial antigens have shown that flagella possess antigenic constituents which are different from those of the body of the organism (*vide* p. 234). It must be recognised, however, that not all cases of motility among the bacteria are dependent on the possession of flagella, for among the spirochætes the movements, which are of various kinds, are due to contractions of the protoplasm of the cell itself. Among most of the higher bacteria also, motility is of similar nature.

The Minuter Structure of the Bacterial Protoplasm.—Many attempts have been made to obtain deeper information as to the structure of the bacterial cell, especially with reference to the differentiation into nucleus and cytoplasm and the intimate phenomena of division. The earliest observations bearing on such points were made on certain large forms, but even with these the minuteness of the cells makes the interpretation of the appearances most difficult. Bütschli, from a study of some large sulphur-con-

taining forms, concluded that the greater part of the bacterial cell may correspond to a nucleus, and that this is surrounded by a thin layer of protoplasm which in the smaller bacteria escapes notice, unless when it can be made out at the ends of the cells. While bacterial protoplasm generally exhibits a selective affinity for nuclear stains, the material thus picked out appears in certain bacteria not to be uniformly distributed through the cell, but to be deposited in certain parts, and controversy has turned on the interpretation of such appearances. Two main views have been held by different schools. Some consider that the bacterial cell contains a formed nucleus and a cytoplasm ; at the same time it is questioned whether all the material giving the reaction of a nucleus is really part of such a central structure and not merely stored material. According to a modification of this view the nucleus is an extended thread lying in the protoplasm—in some bacillary types having a spiral or zigzag appearance. The other view is that the bacterial cell represents a vital unit in which differentiation into nucleus and cytoplasm has not yet occurred, or has become lost, and where the two main elements of higher cells are intermingled with one another, the homologue of the cytoplasm being present in a close meshwork of nuclear material. With regard to the behaviour of the cell in division, among those who hold the former view some have figured appearances in the supposed nucleus which suggest the occurrence of mitosis, but such an interpretation is not generally accepted. It is to be noted, however, that in the vast majority of bacteria nuclear material is so diffused throughout the cell that a " morphological " nucleus cannot be differentiated. We may add that bacteria have been found to contain a considerable proportion of nucleo-protein.

Occasionally irregular, deeply staining granules may be observed in the protoplasm, often, when they occur in a bacillus, giving the latter the appearance of a short chain of minute cocci. These have been called metachromatic granules from the fact that by appropriate procedures they can be stained with one dye, while the rest of the bacterial cell can be made to take on another colour. Such an appearance is well known as occurring in the diphtheria bacillus, especially when stained by Neisser's method (p. 121). In certain bacteria, for example the plague bacillus, the stainable substance is arranged at each pole, leaving an unstained area between —the so-called " polar staining." It is still difficult to interpret the significance of such appearances. They are present in certain bacteria under all circumstances, sometimes they are associated with growth in particular surroundings. Whatever the composition and relationships of the essential parts of the bacterial protoplasm may be, there is, as has been said, reason for believing that even in the lower forms reserve material exists. This may consist of fat, glycogen, and other substances, amongst which may be mentioned volutin, as described by A. Meyer, a substance probably of protein nature characterised by solubility in water, alkalis and acids, and by insolubility in alcohol. It may be concluded that the metachromatic granules are not living derivatives of the bacterial cell and are certainly not of nuclear nature. It has been thought that they might be to some extent artefacts and dependent on plasmolytic changes affecting the cell, but they can be observed by vital staining methods which excludes this possibility.

The Chemical Composition of Bacteria

Corresponding with their natural environment in a fluid medium, the vegetative forms of bacteria contain a large proportion of water, which averages 75 to 85 per cent. The solid constituents show great variations, depending not only on the species under investigation but also on the composition of the culture media, the temperature of growth, and the age of culture; this applies particularly to the inorganic constituents. The proteins of the bacterial cells are composed of the same amino-acids as those of higher forms of life; they include the phosphorus-containing nucleo-proteins and other protein constituents regarding which, however, little is known. Granules formed by certain organisms which show metachromatic staining (volutin granules) are believed to consist of reserve stuffs of protein nature. Fats, lipoids (lecithin and cholesterol, etc.), and waxes form a considerable proportion of bacterial protoplasm; these are specially abundant in the tubercle bacillus and other acid-fast organisms. The influence of the medium in regard to such constituents is well shown by the finding of Eckstein and Soule that *B. coli* grown on a synthetic medium of which alanine was the nitrogenous constituent, contained considerable amounts of phospholipoids, whereas when cystine was substituted only a trace of these was formed. Fat may be intimately incorporated with the bacterial protoplasm, so that extraction methods are required for its demonstration, or at times it may be present as globules stainable by reagents such as osmic acid or Sudan III. Various carbohydrates have been isolated from bacteria, and their capsules are specially rich in such substances, which are of the nature of gums and hemicelluloses; but cellulose is seldom, if ever, detected. A nitrogen-containing carbohydrate allied to chitin is found, and the rigidity of bacteria has been attributed to its presence in the cell wall. Mucin, a compound of protein with carbohydrate, is also formed. Granules of glycogen, which are stained darkly by iodine, are sometimes found in bacteria, also granulose, which is closely related to starch. The mineral constituents include sodium, potassium, magnesium, calcium, and iron salts, as well as traces of other bases, also phosphorus which is always in considerable amount, sulphur, and chlorine. According to the findings of Guillemin and Larson with *B. coli* killed by heating and suspended in water, the greater part of the salts diffuse out of the bacteria. Chlorides, however, diffuse out entirely while the whole of the iron remains fixed to the bodies.

Pigments are produced by many bacteria, although this is not a marked feature in most of the pathogenic organisms. The pigment may be intracellular, as in the case of bacteriopurpurin; sometimes it is extracellular, but usually it is impossible to determine whether the pigment is inside or outside the protoplasm. Often the coloration extends out into the medium for a considerable distance beyond the organisms. Many factors influence pigment formation; thus oxygen is often essential, also suitable conditions as to temperature are required, since an organism which forms pigment at room temperature may fail to do so at 37° C. The composition of the nutrient medium may also be important. Exposure to light tends to intensify pigmentation. Some of the pigments are water-soluble, others are soluble in fat solvents such as chloroform, alcohol, and ether. Certain of the latter are lipochromes allied apparently to carotene, the pigment which is widely distributed in both animals and plants and gives the colour to serum and egg yolk. The chemical composition of bacterial pigments is, however, still to a great extent obscure. A number of organisms form several pigments.

Ferments and toxins are important products to which the action of bacteria is in very great part due; they may either pass into the medium in which the organisms are growing or they may remain intimately attached to the bodies of the bacteria, becoming free only on the disintegration of the latter. Their properties and actions are dealt with later.

Many bacteria form vitamin B, but vitamins A, C, and D are not produced.

The Physical Characters of Bacteria

The superficial layers of a bacterium constitute a definite ectoplasm which tends to act as a semipermeable membrane, so that osmotic phenomena occur. Accordingly, plasmolysis may take place in hypertonic solutions and in hypotonic the cells may burst; the extent of these changes varies, however, with different species and with the age of the cells. In cultures of certain species, e.g. *B. proteus*, bloated and irregular forms are specially apt to occur, and such alterations may result from changing the salt content of the medium either in the direction of decrease or increase (Dunlop and Maitland). Since bacteria are largely composed of hydrophilic colloids, they exhibit the behaviour of such colloid matter in general. In virtue of their minute size also, they present a surface which is large relative to their

volume; therefore they are well adapted to take part in adsorption reactions. In weak solutions of sodium chloride most bacterial cells bear a negative electric charge—that is, they show cataphoresis and migrate to the anode when a direct electric current is passed through the fluid in which they are suspended. By measuring the rate of their migration under the influence of a known electric field, the potential difference between the cells and the fluid may be estimated. The uniform suspension which many species of organisms form in water or weak saline, results from the mutual repulsion of the similarly charged particles, while the molecules of the fluid impart to them active Brownian movements. Under the influence of acids, salts, and certain dyes such bacterial suspensions may exhibit agglutination. Also, a culture of organisms may alter spontaneously in its behaviour and become agglutinated by concentrations of salt which previously did not affect it; this phenomenon is usually accompanied by other alterations, such as the change from a "smooth" to a "rough" type of colony (p. 39).

General Physiology of the Bacteria

There are five prime factors in the life and growth of bacteria which must be considered, namely, food supply, moisture, relation to gaseous environment, temperature, and light.

Food Supply and Metabolism.—In nature bacteria live chiefly on the complex organic substances which are derived from plants and animals or which constitute their dead bodies. As a general rule, many varieties of bacteria grow side by side, so that the food supply of any particular one may depend on the growth of others. But while such *symbiosis* may favour some species, certain organisms are inhibited by the presence of other (*antibiosis*). The production of a disease, however, is usually due to the penetration of one, or at most of a limited number of varieties into the tissues, so that we are concerned chiefly with the growth requirements of isolated species of organisms.

In order that growth may occur, the chemical elements are required which make up protoplasm, and also substances which supply sources of energy. As regards carbon and nitrogen, great differences exist in the materials from which different bacteria can supply their needs. Certain bacteria, like the higher plants, can assimilate carbon dioxide while utilising nitrogen from an inorganic source; these are "autotrophic."

As a rule bacteria require their sources of carbon to be in a more reduced form than carbon dioxide—such organisms are called "heterotrophic"; the pathogens are included in this group. Again, the nitrogen of the air is assimilated by certain soil bacteria and others use nitrites, but this is exceptional. The degradation products of protein, such as peptones, polypeptides and amino-acids, are very widely utilised; the amides of organic acids, *e.g.* asparagine, may also serve as a source of nitrogen to the heterotrophic species. Complete proteins as a rule do not appear to be utilised as food-stuffs. Many heterotrophic organisms, however, can use the nitrogen of ammonia salts to synthesise essential amino-acids, particularly tryptophane, provided a suitable organic source of carbon is present; these may be termed "non-exacting" as regards their nitrogen supply. Others are "exacting" and can only utilise more complex sources of nitrogen such as amino-acids. Even in a given species, e.g. *B. typhosus*, while the majority of strains are exacting, certain ones are non-exacting and can utilise ammonia. But by "adaptation" or "training" bacteria may acquire the power of assimilating or breaking down substances either nitrogenous or carbonaceous which they did not originally act upon. For instance, an exacting strain of *B. typhosus* can be induced to grow in a medium lacking tryptophane and to synthesise this compound. Such changes, in some cases at least, seem to depend on the organisms acquiring the power to form "adaptive" enzymes which possess an affinity for the substrates in question. While carbon is also provided by the more complex nitrogenous compounds, carbohydrates are in addition extensively utilised as food-stuffs; these are fermented and this process probably serves the double purpose of liberating energy and of providing the most suitable types of carbonaceous compounds for assimilation. Salts of organic acids, *e.g.* citrates, lactates, etc., may serve as a source of carbon to some species. According to Quastel, pyruvic acid is the form in which carbon is assimilated by *B. coli*, and a carbonaceous substance to be utilised must be convertible by the bacterial enzymes into this compound. The tubercle bacillus finds in glycerol a particularly suitable source of carbon. In the case of organisms which grow in the presence of air, energy is obtained usually by oxidations.

As regards mineral constituents, potassium or sodium and phosphorus, chlorine, sulphur and carbon—in the form of phosphates, chlorides, sulphates and carbonates—would appear to be the only essentials for many organisms, while others may require also magnesium, calcium, or iron; but it is difficult to say how

far minute traces of other minerals are of importance. The sulphur bacteria assimilate sulphuretted hydrogen and store in their bodies granules of sulphur which disappear when the organisms are starved.

In general a solution of the products of tryptic digestion of meat affords a suitable medium for many organisms, including the pathogenic varieties. While a large number of organisms will grow indefinitely on synthetic media of known composition, there are some which cannot be cultivated under these conditions, e.g. *Streptococcus pyogenes*, pneumococcus, and Pfeiffer's bacillus. It would appear that in the case of these, accessory food factors are required. These accessory principles can be derived from various sources, *e.g.* by fractionation of a watery extract of rice bran, and it is important that organisms which are capable of flourishing on synthetic media, themselves produce the growth-promoting substances. Pfeiffer's bacillus requires two accessory factors, one of which resembles a vitamin. It is doubtful, however, whether such growth factors are identical with any of the vitamins required by higher animals Regarding the question of the requirement by bacteria of vitamin-like substances, Knight and Fildes have demonstrated certain accessory principles for growth which act in such exceedingly minute amounts as to place them in the category of vitamins. A substance of this nature necessary for the growth of *B. sporogenes* is present in yeast, urine, etc., and an analogous principle is required by *Staphylococcus aureus*. This latter can be separated from marmite and to some extent purified by chemical procedures.

Certain highly parasitic bacteria, *e.g.* gonococcus, meningococcus, etc., fail to grow in the absence of blood-serum, and the growth of many pathogenic species is promoted in the presence of serum. The bacillus of Johne's disease exhibits a peculiar requirement, viz. some substance synthesised by other acid-fast bacteria; thus dead tubercle bacilli or timothy grass bacilli added to the medium enable it to flourish. It should be noted in regard to culture media generally, that filtration through paper, cotton wool, etc., may by adsorption remove essential accessory food substances.

Most bacteria seem to form products which are unfavourable to their own vitality, for when a species is sown on a mass of nutrient medium it soon ceases to grow, even before the food supply is exhausted; alteration in hydrogen-ion concentration explains only in part the cessation of growth. It should be noted also that substances such as many of the amino-acids,

when present in excess, tend to inhibit growth. When the food supply of a bacterium fails, it degenerates and dies. The proof of death lies in the fact that when it is transferred to fresh suitable media it does not multiply. If the bacterium forms spores it may survive the want of food for a very long time.

The reaction (H-ion concentration) of the food medium is a matter of great importance. Most bacteria prefer a slightly alkaline medium, and some, *e.g.* the cholera vibrio, will not grow in the presence of the smallest amount of free acid; but on the other hand some, like the lactic acid organisms, are highly tolerant of an acid reaction.

A distinction must be drawn between the conditions in media which promote the maximal growth of organisms and those which lead to the longest persistence of vitality. In this connection it is noteworthy that certain organisms, *e.g.* gonococcus and pneumococcus, die quickly when suspended in a simple saline solution (0·85 per cent. NaCl).

Moisture.—The presence of water is necessary for the continued growth of all bacteria. The amount of drying which bacteria in the vegetative stage will resist varies very much in different species. Thus the cholera vibrio is killed by two or three hours' drying, while the *Staphylococcus aureus* will survive ten days' drying, and the *Bacillus diphtheriæ* still more. In the case of spores the periods are much longer. Anthrax spores will survive drying for several years if kept in the dark and exposed to air. While the above statements apply to drying as ordinarily carried out, intense rapid drying preserves the vitality of many vegetative forms for long periods, although some, *e.g.* gonococcus, die rapidly.

Relation to Gaseous Environment.—The relation of bacteria to the oxygen of the air is such an important factor in the life of bacteria that it enables a biological division to be made among them. Some bacteria live and grow best when there is free access of oxygen from the air. To these the title of *aerobes* is given. Other bacteria will only grow when free oxygen is absent; they require also a medium which possesses a suitable degree of reducing action. These are called *anaerobes*; but different species show a wide range in their intolerance of oxygen. In still other bacteria the amount of oxygen is a matter of indifference within wide limits; such organisms are usually denominated *facultative anaerobes*—being aerobic but capable of growing with very little oxygen. Examples of aerobes are *B. tuberculosis* and *B. subtilis*; of a strict anaerobe, *B. tetani*, while the great majority of pathogenic bacteria are

facultative anaerobes. Those bacteria which flourish best in the presence of a minute trace of oxygen, as is shown by the fact that, in a deep culture, growth is most abundant at a point a short distance below the surface, are designated *micro-aerophilic*. In the absence of oxgyen the luminescent bacteria isolated from sea water cease to give out light.

The respiratory mechanisms of bacteria have attracted much attention recently, particularly in relation to the processes of cellular oxidation and reduction. Observations have demonstrated the complexity of the mechanisms involved; and it must be noted that bacteria vary widely in their oxidative processes, and even with any one species, variations may occur according to the conditions of life and growth. When bacteria are growing in a culture medium such as broth, it is usually found that there is a reducing tendency in the system which is greater than that of the broth itself. This has long been known from the fact that reducible substances, such as dyes, when introduced into fluid cultures are often converted into the colourless leuco products, the original colour being restored by vigorous oxygenation. The oxygen which organisms obtain by means of their metabolic processes appears to serve the purpose of oxidising nutrient materials in the medium which are required for life and proliferation. Accordingly, there co-exist in a culture reactions of oxidation and reduction which give rise to changes in electrical potential. The intensity of these changes can be measured by an apparatus illustrated in Fig. 1.

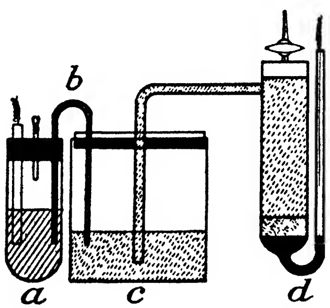


Fig. 1.—Arrangement of cell for measuring oxidation-reduction potentials.

Into the fluid culture medium in the vessel *a* there dips an unalterable electrode consisting *e.g.* of platinum foil. Also dipping into the culture medium is one end of a U-tube *b* filled with agar made up with a saturated solution of KCl. The other end dips into the vessel *c* also containing saturated KCl solution; *c* communicates by a tube containing the same solution with a "standard half cell" *d* consisting of a calomel electrode whose composition is shown. Wires leading from the platinum electrode and the mercury of the standard half cell

are connected to a potentiometer and galvanometer calibrated to ± 1 volt and reading to the nearest millivolt. In this way, the potential of the standard half cell being known, relative to that of the hydrogen electrode, the potential of the system can be measured in volts. This is the electrode potential, Eh ; it is negative when the system is a reducing one and positive when it is oxidising. Another method of estimating Eh depends on the fact that a number of dyes, including indophenols, toluylene blue, thionin, methylene blue and indigo derivatives, change colour on reduction over different Eh ranges and so can be used as indicators. Their use is attended by various difficulties, however, since they may themselves participate in the oxidation-reduction reactions ; also they may exert catalytic or toxic effects ; further, their colour frequently depends on the pH as well as the Eh.

By following the behaviour of Eh in cultures at different stages of growth a number of interesting observations have been made, the full significance of which has not yet been determined. Thus, Hewitt has shown that streptococci and pneumococci differ in their behaviour from staphylococci and diphtheria bacilli. With the two former the Eh begins to rise at once after active growth has ceased, while with the latter at the same stage it remains low. The effect of aeration of the cultures varies also. With streptococci and pneumococci the Eh falls very much less than in unaerated cultures, while with staphylococci and diphtheria bacilli aeration produces little difference. The high level of Eh reached by streptococci and pneumococci corresponds with the formation of hydrogen peroxide.

In the case of aerobic organisms growing under certain conditions free oxygen is utilised and carbon dioxide is given off (Stephenson and Whetham), but apart from atmospheric oxygen other substances may serve as oxygen donators for the oxidative processes of the bacteria. Thus the energy requirements of anaerobic organisms appear to be supplied by the decomposition of such substances as glucose which can be transformed into lactic acid with the production of energy in the absence of free oxygen. It should be noted that oxidation is always accompanied by a corresponding process of reduction. The withdrawal of oxygen from one substance to another implies the reduction of the former, the donator ; likewise the transfer of hydrogen involves the oxidation of the donator with the reduction of the acceptor.

In the case of the anaerobe *B. sporogenes*, such oxido-reduction between pairs of amino acids yields energy for purposes

of nutrition (Strickland reaction). Some organic substances are very readily oxidised in the presence of free oxygen, a peroxide being formed which when acted on by a peroxidase yields active oxygen for the oxidation of other substances which are not auto-oxidisable. But other possible mechanisms depend on substances which readily pass from the oxidised to the reduced state and *vice versa*. Such are glutathione (Hopkins) and the respiratory pigment system, cytochrome (Keilin) which is related to hæmatin, and the yellow oxidation enzyme (Warburg and Christian). It is doubtful if the first of these occurs in bacteria, but cytochrome is widely found in aerobes though not in pneumococci and streptococci nor in anaerobes, and Warburg's pigment is also a constituent of cells in general. Pyocyanin, the pigment of *B. pyocyaneus*, also probably has a respiratory function.

According to McLeod and Gordon, the anaerobic character of organisms, such as *B. tetani* or *B. welchii*, depends on the fact that in cultures in the presence of oxygen they form peroxide of hydrogen. Certain aerobic bacteria also form this substance, but in virtue of the catalase which they produce at the same time they are protected from the peroxide, which is thus destroyed before it can accumulate in an amount sufficient to prove harmful. *B. tetani* and other anaerobes fail to form catalase, and as they are extremely susceptible to peroxide, growth cannot go on in the presence of air. According to Knight and Fildes the spores of *B. tetani* cannot germinate if the Eh is higher than about +0·1 volt; and for growth of the strict anaerobes the Eh must have a value between −0·006 to −0·436 volt. Recent work of O'Meara has indicated that in the case of an anaerobic organism such as *B. tetani*, the harmful effect of oxygen is intensified by the presence in the medium of traces of certain copper compounds.

With regard to anaerobes, hydrogen and nitrogen are indifferent gases. Many anaerobes, however, do not flourish well in an atmosphere of carbon dioxide. *B. abortus*, on the other hand, when freshly recovered from the tissues, requires for its growth the presence of a considerable excess of carbon dioxide. Also, according to the observations of Gladstone, Fildes, and Richardson, the presence of carbon dioxide is essential for the growth of many pathogenic organisms. Few experiments have been made on the action on bacteria of gases under pressure. Increased pressures of carbon-dioxide and of oxygen both prevent the proliferation of *B. pyocyaneus*; but whereas the former soon proves lethal, very high pressures of oxygen are merely

inhibitory and growth occurs subsequently on returning the culture to atmospheric pressure of air. The anthrax bacillus again is readily killed by exposure either to oxygen or carbon dioxide at slightly increased pressures, while many coliform bacilli will survive high pressures of both. Hydrogen at high pressures is not harmful.

Temperature.—For every species of bacterium there is a temperature at which it grows best. This is called the "optimum temperature." There is also in each case a maximum temperature above which growth does not take place, and a minimum temperature below which growth does not take place. As a general rule the optimum temperature is about the temperature of the natural habitat of the organism. For organisms taking part in the ordinary processes of putrefaction the temperature of warm summer weather (20° to 24° C.) may be taken as the average optimum, while for organisms normally inhabiting animal tissues 35° to 39° C. is a fair average. The lowest limit of ordinary growth is from 12° to 14° C., and the upper is from 42° to 44° C. In exceptional cases growth may take place as low as 5° C., and as high as 70° C. Some organisms which grow best at a temperature of from 60° to 70° C. have been isolated from animal manure, the intestinal tract, etc. These have been called *thermophilic* bacteria. It is to be noted that while growth does not take place below or above a certain limit, it by no means follows that death takes place outside such limits. Organisms can resist cooling below their minimum or heating beyond their maximum without being killed. Their vital activity is merely paralysed. Especially is this true of the effect of cold on bacteria. The results of different observers vary; but if we take as an example the cholera vibrio, Koch found that while the minimum temperature of growth was 6° C., a culture might be cooled to −32° C. without being killed. When kept immersed in liquid air, *B. typhosus* and *Staphylococcus aureus* were still living after six months (Macfadyen and Rowland). With regard to the upper limit, harmful effects are usually observed a few degrees above the optimum, and ordinary organisms in a spore-free condition will seldom survive a temperature of 55° C., if long enough applied. Many organisms lose some of their properties when grown at unnatural temperatures. Thus many pathogenic organisms lose their virulence if grown above their optimum temperature, and some chromogenic forms, most of which prefer rather low temperatures, lose their capacity of producing pigment, e.g. *Spirillum rubrum*.

Effect of Light.—Much attention has been paid to this factor in the life of bacteria. Direct sunlight is found to have a very inimical effect. It has been found that an exposure of dry anthrax spores for one and a half hours to sunlight kills them. When they are moist, a much longer exposure is necessary. Typhoid bacilli are killed in about one and a half hours, and similar results have been obtained with many other organisms. In such experiments the thickness of the medium surrounding the growth is an important point. Death takes place more readily if the medium is scanty or if the organisms are suspended in water. Any fallacy which might arise from the effect of the heat rays of the sun has been excluded, though light *plus* heat is more fatal than light alone. In direct sunlight it is chiefly the green, violet, and the ultra-violet rays which are fatal. The last-mentioned rays, however produced, have a powerful bactericidal action. By using a quartz spectrometer with a tungsten arc, Browning and Russ showed that the ultra-violet rays with bactericidal action occupy a position in the spectrum at some distance from the visible rays—from 2960 to nearly 2100 Ångström units. The exact extent varies somewhat in the case of different organisms, but the area of rays in the spectrum effective against any one organism is comparatively sharply marked off. The bactericidal rays have little penetrating power, being completely absorbed by human skin in a thickness of 10 mm. These observers also found that those, and only those, rays which are bactericidal to the *Staphylococcus aureus* are absorbed by an emulsion of that organism. Diffuse daylight has also an injurious effect upon bacteria, though it takes a much longer exposure to do serious harm. A powerful electric light is as fatal as sunlight. Here, as with other factors, the results vary very much with the species under observation. Another effect of light, which, however, does not account for all the above results, is to produce peroxide in uninoculated medium, which then acts as an antiseptic. The formation of peroxide appears also to account for the photo-dynamic action of certain dyes, *e.g.* eosin, which when added to a suspension of bacteria, rapidly causes their death on exposure to light, provided that oxygen is present. Bacteria are killed by exposure to X-rays of high intensity only. The purple bacteria are the only members of the group in which assimilation is aided by exposure to light, either in the visible range or infra-red ; this is due to their pigment, which contains a constituent closely related to chlorophyll.

Conditions affecting the Movements of Bacteria.—In some

cases differences are observed in the behaviour of motile bacteria, contemporaneous with changes in their life-history. Thus, in the case of *B. subtilis*, movement ceases when sporulation is about to take place. On the other hand, in the bacillus of symptomatic anthrax (*B. chauvœi*), movement continues while sporulation is progressing. Under ordinary circumstances motile bacteria appear not to be constantly moving, but occasionally to rest; the movements become more active if the temperature be raised. In certain species of bacteria which are typically motile, e.g. *B. typhosus*, non-motile strains may exist; this is not invariably associated with the absence of the flagella, however. Motility can be induced in such flagellated cultures by growth of the organisms on semi-solid media. Most interest however, attaches to the fact that bacilli may be attracted to certain substances and repelled by others. Schenk, for instance, observed that motile bacteria were attracted to a warm point in a way which did not occur when the bacteria were dead and therefore subject only to physical conditions. Most important observations have been made on the attraction and repulsion exercised on bacteria by chemical agents, which have been denominated respectively *positive* and *negative chemotaxis*. Pfeffer investigated this subject in many unicellular organisms. The method used was to fill with the agent a fine capillary tube closed at one end, to introduce this into a drop of fluid containing the bacteria under a cover-glass, and to watch the effect through the microscope. The general result was to indicate that motile bacteria may be either attracted or repelled by the fluid in the tube. The effect of a given fluid differs in the case of different organisms, and a fluid chemotactic for one organism may not act on another. Degree of concentration is important, but the nature of the fluid is more so. Of inorganic substances, salts of potassium are the most powerfully attracting. Further, solutions of peptone and meat extract have powerful chemotactic properties. Carbohydrates in solution have little effect, and glycerol is neutral. Acids, alkalis, and alcohol are repellent. Corresponding chemotactic phenomena are shown also by certain animal cells, *e.g.* leucocytes, to which reference is made below.

The Parts played by Bacteria in Nature.—As has been said, the chief effect of bacterial action in nature is to break up into more simple combinations the complex molecules of the organic substances which form the bodies of plants and animals, or which are derived from them. That the very complicated process of putrefaction is due to bacteria was first proved by

Pasteur, for any organic substance can be preserved indefinitely from ordinary putrefaction by the adoption of some method of killing all bacteria present in it, as will be afterwards described. Bacteria, as well as yeasts and higher fungi, are responsible also for the analogous processes of breaking down of carbohydrates which are comprised in the term fermentation. Bacteria may produce alcoholic, lactic, and butyric fermentations in addition to other types. Such processes may be wasteful, as in the spoilage of milk and the development of rancidity in butter, or they may be economically important, *e.g.* in the ripening of cheese or the manufacture of acetone. The disintegration of organic material, which is so prominent an effect of bacterial growth, is, of course, an accompaniment of the synthesis of the complex substances of which the bacteria themselves are built up. The most striking example of such synthetic power is presented in the case of the bacteria which in the soil make nitrogen more available for plant nutrition by converting ammonia into nitrites and nitrates. Winogradsky, by using media containing non-nitrogenous salts of magnesium, potassium, and ammonium, and free of organic matter, demonstrated the existence of forms which convert, by oxidation, ammonia into nitrites, and of other forms which convert these nitrites into nitrates. Both can derive their necessary carbon from alkaline carbonates. Other bacteria can actually take up and combine into new compounds the free nitrogen of the air. These are found in the tubercles which develop on the rootlets of the leguminosæ (*B. radicicola*). Without such organisms the tubercles do not develop, and without the development of the tubercles the plants are poor and stunted. Bacteria thus play an important part in the enrichment and fertilisation of the soil.

A certain comparatively small number of bacteria have been proved to be the causal agents in some disease processes occurring in man, animals, and plants. This means that the fluids and tissues of living bodies are, under certain circumstances, a suitable habitat for the bacteria involved. The processes by which the disease effects are produced will be studied in detail later. Meantime we may note that the disease-producing effects of bacteria form the basis of another biological division of the group. Some bacteria are harmless to animals and plants, and apparently under no circumstances give rise to disease in either. These are known as *saprophytes*. They are normally engaged in breaking up dead animal and vegetable matter. Others normally live on or in the bodies of plants

and animals. These are known as *parasitic bacteria*. Certain of these produce specific diseases: others exist on body surfaces (skin and mucous membranes) without, under ordinary circumstances, exerting harmful effects. Sometimes an attempt is made to draw a hard-and-fast line between the saprophytes and the parasites, and obligatory saprophytes or parasites are spoken of. This is an erroneous distinction. The fact that most bacteria associated with disease processes, and proved to be the cause of the latter, can be grown in artificial media, shows that such parasites can be adapted to a saprophytic existence.

The Methods of Bacterial Action.—The processes which organic substances undergo in being split up by bacteria depend, first, on the chemical nature of the substances involved, and, secondly, on the varieties of the bacteria which are acting. For an exact knowledge of the capacities of any particular bacterium there must be an accurate chemical examination of its effects when it has been grown in artificial media the nature of which is known. The destruction of protein substances which is mostly involved in the wide and varied process of putrefaction, can be undertaken by different varieties of bacteria. The action of the latter is analogous to what takes place when proteins are subjected to gastric and intestinal digestion. In these circumstances, therefore, the production of albumoses, peptones, etc., similar to those of ordinary digestion, can be recognised in putrefying solutions, though the process of destruction always goes further, and there results a mixture of simpler substances, including fatty acids and bases, ammonia, carbonic acid, methane, sulphuretted hydrogen and other foul-smelling gases, and indole. The process is an exceedingly complicated one when it takes place in nature, and different bacteria (especially anaerobes) are concerned in the different stages. An indication of slight proteolytic action is afforded by liquefaction of gelatin, fibrin, or coagulated serum or egg-white; such properties are exhibited by a number of pathogenic organisms. When carbohydrates are split up, various alcohols, esters, and acids are produced. One common result of bacterial action is thus an alteration of the reaction of the medium, sometimes towards the acid, sometimes towards the alkaline side. A reversal of reaction may occur, *e.g.* acid substances being produced at first, and these then being broken down further with the formation of less acid products. Reduction and oxidation phenomena are also frequently observed. The formation of ammonia from nitrites and of the latter from

nitrates by organisms in the soil are important examples of reduction. The reduction of certain other types of substances such as methylene blue or potassium di-indigo disulphonate appears to depend on a different mechanism. Organisms which have this property tend also to form peroxide, and so are exposed to the bactericidal action of the latter unless they produce catalase, which prevents its accumulation. Oxidations are effected by many bacteria; one instance is the conversion of hæmoglobin into methæmoglobin by the pneumococcus when growing on blood agar. Many substances are produced by bacteria, of the exact nature of which we are still ignorant; for example, the *toxins* which play such an important part in the action of many pathogenic species (p. 189).

The chemical actions of bacteria depend on the production by them of *ferments* of a very varied nature and complicated action. Thus the liquefaction of gelatin or coagulated serum is due to a proteolytic enzyme which passes into the medium in which organisms are growing. Ferments which invert sugar, which split up sugars into alcohols or acids, which coagulate casein, which form ammonium carbonate from urea, also occur.

Such ferments may diffuse readily into the surrounding fluid (frequently as a result of autolysis of the organisms), or they may be retained in the cells where they are formed. In the latter case the bacterial protoplasm often must be thoroughly disintegrated, *e.g.* by grinding, before the ferment is liberated. That a purely intracellular digestion may take place is illustrated by what has been shown to occur in the case of the *Micrococcus ureæ*, which from urea forms ammonium carbonate. Here, if after the action has commenced the bacteria are filtered off, no further production of ammonium carbonate takes place, which shows that no ferment has been dissolved out into the urine. If now the bodies of the bacteria be extracted with absolute alcohol or ether, either of which of course destroys their vitality, a substance is obtained of the nature of a ferment, which, when added to sterile urine, rapidly causes the production of ammonium carbonate. This ferment has evidently been contained within the bacterial cells. In the investigation of the phenomena of the ferment action of bacteria, it has been noted in certain cases that the ferments formed depend on the substrate offered to the bacterium. Thus in one case a bacterium growing in starch forms diastase, but it does not do so when grown on sugar.

The Classification of Bacteria

In what we have to say under this heading we shall chiefly confine ourselves to the characters of the pathogenic bacteria. In the past there have been numerous schemes set forth for the classification of bacteria, the fundamental principle running through all of which has been the recognition of the two subdivisions and the type-forms mentioned in the opening paragraphs above. There has been little agreement, however, among systematists as to the characters on which more detailed classification should be based, and even yet our knowledge of the essential morphology and relations of the bacteria is too limited for an exact classification on a strictly biological basis. Identification of species is dependent, not only on the morphology of individual organisms and that of growths on culture media, but also on physiological and bio-chemical characters, pathogenicity to animals under experimental conditions, and in some cases on delicate serum reactions. In recent years an extensive scheme of classification has been put forward by American systematists, and this will be referred to below after a simple general account has been given.

The division into lower and higher bacteria can be recognised, though transitional forms have to be accounted for. In subdividing the bacteria further, the morphological forms they present constitute a broad basis of classification. The lower bacteria thus naturally fall into the four morphological groups mentioned, (1) cocci, (2) bacilli, (3) spirilla, and (4) spirochætes. Subsidiary, though important, points in the further subdivision are the planes in which fission takes place (among the cocci). the presence or absence of spores, etc. The recognition of actual species is often a matter of great difficulty. The points to be observed in this will be discussed in connection with the individual organisms.

I. The Lower Bacteria.[1] — These, as we have seen, are minute unicellular masses of protoplasm surrounded by an envelope, the total vital capacities of a species being represented in every cell. They present four distinct forms just mentioned. Endogenous sporulation may occur; they may also be motile.

1. *Cocci* (or *Micrococci*).—In this group the cells range in different species from 0·5 μ to 2 μ in diameter, but most measure about 1 μ. Before division they may increase in size in all directions. The main groups are usually classified according to

[1] For the illustration of this and the succeeding systematic paragraphs, see Fig. 2.

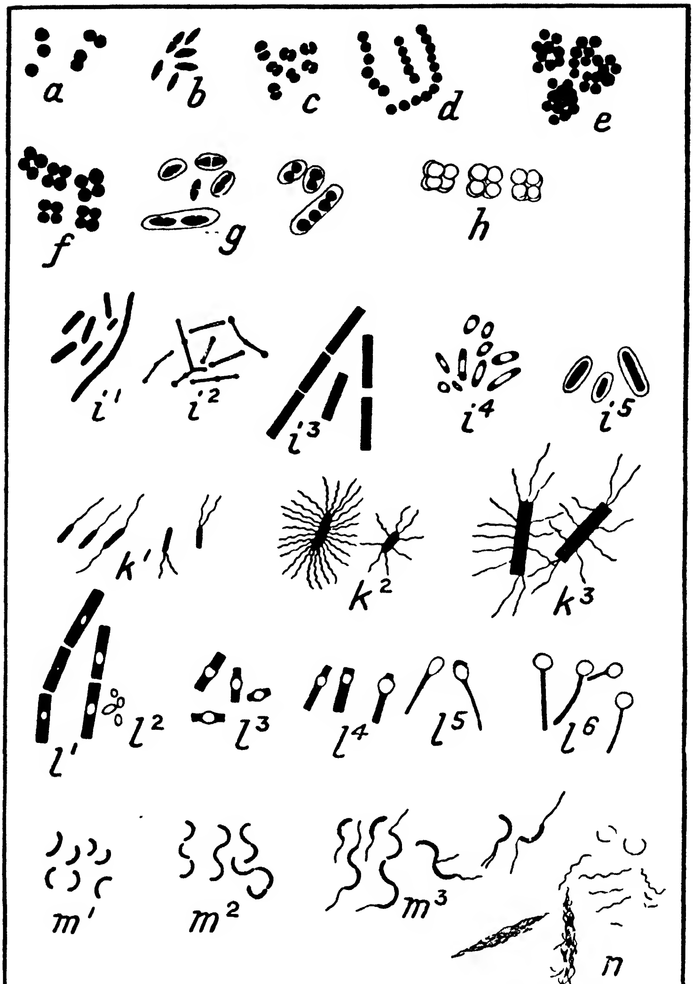
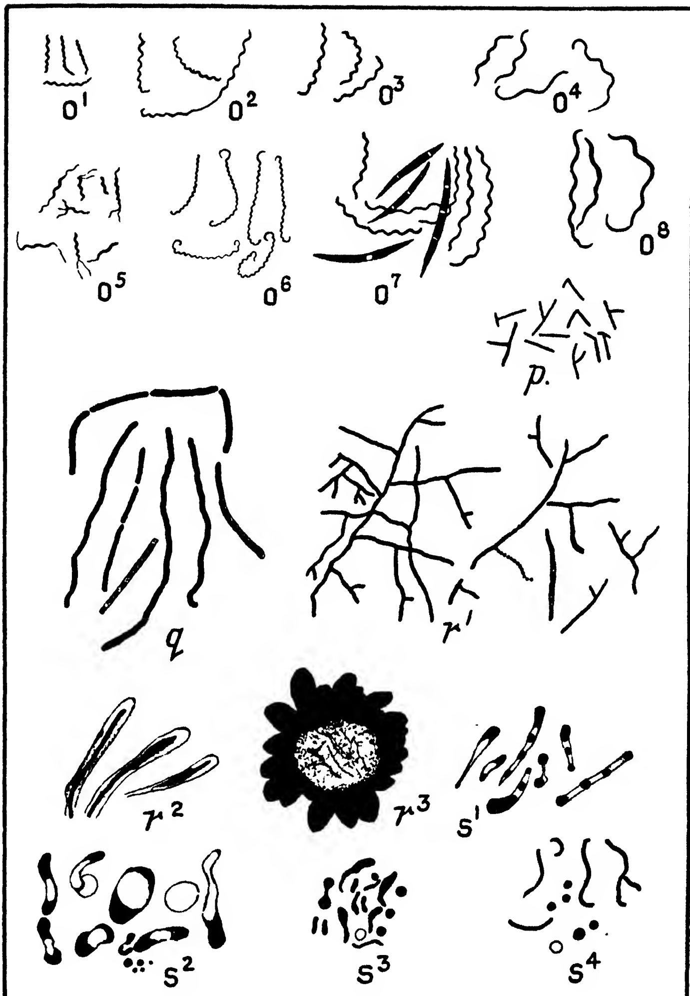


FIG. 2.—*a–h*. Different types of cocci. *a*. Single round cocci and simple diplococcal forms. *b*. Lancet-shaped cocci (pneumococcus). *c*. Kidney-bean-shaped cocci (gonococcus). *d*. Streptococci. *e*. Staphylococci. *f*. Tetrads (*Micrococcus tetragenus*). *g*. Capsulated cocci (pneumococcus). *h*. Sarcina forms. i^1–i^6. Bacilli. i^1. Simple rod forms (*Bacillus coli*). i^2. Diphtheroid type. i^3. Streptobacillus (*B. anthracis*). i^4. Bacilli with vacuolated protoplasm (*B. pestis*—Pasteurella group). i^5. Capsulated bacilli. k^1–k^3. Different forms of flagellated bacilli. k^1. Terminal flagellated forms. k^2, k^3. Peritrichous forms (k^2. *B. typhosus;* k^3. *B. subtilis*). l^1–l^6. Spore-bearing types of bacilli. l^1. Central spores (*B. anthracis*). l^2. Free spores (*do.*). l^3. Central projecting spores. l^4. Subterminal spores. l^5. Terminal spores of oval type. l^6. Terminal spores of spherical type (*B. tetani*). m^1–m^3. Vibrio forms. m^1. Comma form. m^2. Spirillum form. m^3. Flagellated forms. *n*. Detached flagella—isolated and in whorls.

a. *h.* ×2000 · *i.*–*n.* ×1500.



(Fig. 2 *continued*)—

o^1–o^8. Spirochætal forms. o^1–o^3. Treponema forms (o^1. *Tr. microdentium*; o^2. *Tr. pallidum*; o^3 *Tr. gracile*). o^4. Borrelia type (*Borrelia refringens*). o^5. Spirillum (*Sp. minus*). o^6. Leptospira. o^7. Spirochætes along with fusiform bacilli (from Vincent's angina). o^8. Large spirochæte (from mouth, of *Borrelia refringens* type). *p*. Branched bacilli (*B. bifidus*). *q*. Leptothrix. r^1–r^3. Streptothrix forms. $r.^1$. Streptothrix. r^2. *do.*, showing filaments with sheath. r^3. *Actinomyces*—small colony from tissues showing clubs. s^1–s^4. Involution forms. s^1. *Bacillus diphtheriæ*. s^2. *Bacillus pestis*. s^3. *Bacillus influenzæ*. s^4. *Vibrio choleræ*.

o.–s. × 1000.

the method of division. If the cells divide only in one axis, and through the consistency of their envelopes remain attached, then a chain of cocci will be formed. A group in which this occurs is known as a *Streptococcus*. If division takes place irregularly, the resultant mass may be compared to a bunch of grapes, and the group is often called a *Staphylococcus*. Division may take place in two axes at right angles to one another, in which case cocci adherent to each other in plates of four (called tetrads) or multiples of four may be found, the former number being the more frequent. The individuals in a growth of micrococci often show a tendency to remain united in twos. This form is spoken of as a *Diplococcus*, but may not be distinctive, since every coccus as a result of division becomes a diplococcus, though in some species the tendency to remain in pairs is well marked. The adhesion of cocci to one another depends on the character of the capsule. Often this has a well-marked outer limit (*Micrococcus tetragenus*), sometimes it is of great extent, its diameter being many times that of the coccus (*Streptococcus mesenteroides*). In none of the cocci have endogenous spores been certainly observed. Usually included in this group are coccus-like organisms which divide in three planes at right angles to one another. This form is referred to as a *Sarcina*. Usually they are seen in cubes of eight with the sides which are in contact slightly flattened. Large numbers of such cubes may be lying together. The *Sarcinæ* are, as a rule, rather larger than the other members of the group. Most of the cocci are non-motile, but a few motile species possessing flagella have been described; possibly, however, these are more of the nature of short bacilli—cocco-bacilli.

2. *Bacilli.*—These consist of long or short cylindrical cells, with rounded or sharply rectangular ends, usually not more than 1 μ broad, but varying very greatly in length. They may be motile or non-motile. Where flagella occur, these may be distributed all round the organism—*peritrichous*, or only at one or both of the poles—*terminal*. Several species are provided with sharply-marked capsules (*e.g.* pneumobacillus). In many species endogenous sporulation occurs. The spores may be central, terminal, or subterminal, round, oval, or spindle-shaped. There is no doubt that among the bacilli in certain cases, *e.g.* in *B. diphtheriæ* and *B. tuberculosis*, the phenomenon of true branching may occur. Such instances form a connecting link between the bacilli and the higher bacteria, *e.g.* *Streptothrices*.

3. *Vibrios* and *Spirilla*.—These consist of curved rods or

cylindrical non-flexuous cells more or less spiral or wavy. The unit is usually a short curved rod or *vibrio* (often referred to as a "comma" form). When two or more of the latter occur, as they often do, end to end with their curves alternating, then a wavy or spiral thread results. An example of this is the cholera microbe (Fig. 2, m^2). The motile species possess terminal flagella. Of the latter there may be one or two, or a bunch containing as many as twenty, at one or both poles (Fig. 2, m^3). Division by transverse fission takes place as among the bacilli.

4. *Spirochætæ.*—These are elongated flexuous cells which show undulations or regular spirality. As a rule they are slender in proportion to their length, and some of the smaller forms are of extreme tenuity. Motility is due to contraction of the protoplasm and may be of a lashing, wave-like, oscillatory, or corkscrew-like character. Some are tapered at their extremities to a flagellum-like structure. The larger forms reach a considerable size and a relatively great length, and some of them have a spirally-twisted crest running along the whole length of the cell. They multiply by transverse fission, but longitudinal division has been described in some of them, although this is doubtful. The pathogenic species belong to different genera—*Treponema*, e.g., organisms of syphilis and yaws, etc., *Borrelia*, e.g., organisms of relapsing fever, and *Leptospira* (organisms of infective jaundice).

II. The Higher Bacteria.—These consist of definite filaments, branched or unbranched. In most cases the filaments at more or less regular intervals are cut by septa into short rod-shaped or curved elements. Such elements are more or less interdependent, and special staining methods are often necessary to demonstrate the septa which demarcate the individuals of a filament. There is further often a definite membrane or sheath common to all the elements in a filament. Not only, however, is there this close organic relationship between the elements of the higher bacteria, but there is also interdependence of function; for example, one end of a filament is frequently concerned merely in attaching the organism to some other object. The greatest advance on the lower bacteria, however, consists in the setting apart among some of the higher bacteria of the free terminations of the filaments for the formation of *conidia*; groups of such conidia may be formed by division in three different planes, for the production of new individuals, as has been described (p. 3).

The conidia have a free existence for a certain time before becoming attached, and in this stage are sometimes motile.

They are usually oval or rod-like, sometimes pyriform. They do not possess any special powers of resistance.

The following may be given as a brief summary of the main orders: (*a*) The *Chlamydobacteria* or *Trichobacteria* are filamentous forms, occurring mainly in water, and are usually sheathed; the sheath is often impregnated with iron oxide. They are often sessile, and the attached ends thinner than the distal. The *Leptothrix* which is a common commensal of the mouth, belongs to this order. Conidium formation is often met with in the free ends of the filaments; the conidia may be motile or non-motile. True branching does not occur, but false branching is sometimes met with, due to a terminal cell becoming laterally displaced, *e.g.* in *Cladothrix*. (*b*) A further development occurs in the *Streptothrices*, to which the *Streptothrix actinomyces* and other pathogenic species belong. Here the organism consists of a felted mass of non-septate filaments in which true dichotomous branching occurs. Under certain circumstances threads grow out and produce chains of conidia, from which new individuals can be reproduced. These conidia are sometimes spoken of as spores, but they have not the same staining reactions nor resisting powers of so high a degree as the endospores of bacteria. Sometimes, too, the protoplasm of the filaments breaks up into bacillus-like elements which may also have the capacity of originating new individuals. In the *Streptothrix actinomyces* a club-shaped swelling at the end of the filament may occur; this is probably a product of a degenerative change or possibly of defensive nature. The *Streptothrix* group may be regarded as a link between the bacteria on the one hand and the lower fungi on the other. Like the latter it shows the felted mass of branching filaments which is usually called a mycelium, whilst the breaking up of the protoplasm into coccus- and bacillus-like forms links it to the other bacteria. The *Streptothrices* are put by some writers amongst the *Chlamydobacteria*. (*c*) The *Thiobacteria* or sulphur bacteria are characterised by the presence of sulphur granules in their protoplasm: bacterio-purpurin also may be present. Some of them are sheathed and attached and form conidia, e.g. *Thiothrix*; others again are elongated, sheathless, and show an undulatory motion due to the contraction of the protoplasm, e.g. *Beggiatoa*.

New Classification and Nomenclature.—In recent years, owing to the unsatisfactory state of bacterial classification and nomenclature, an attempt has been made by American systematists to develop a new system of classification in accordance with that adopted in other branches of biological study. The classification

advocated seems sufficiently elastic to provide for future modification as our knowledge of the relationships of the bacteria is added to. This system introduces a new nomenclature and eliminates the trinomial and clumsy designations that have crept into bacteriology, but it seems doubtful whether new names should be applied to certain well-known pathogenic organisms which already possess a convenient designation recognised by long usage. Such terms as gonococcus, meningococcus, *Bacillus typhosus*, etc., are likely to maintain their traditional use in preference even to designations that clearly indicate the approved biological genus and species of the organisms. The new classification, however, serves a most helpful purpose in its orderly grouping together of biologically allied species.

The bacteria are collectively designated as the class of *Schizomycetes* which is defined in a general way as follows: minute, unicellular organisms, chlorophyll-free, usually colourless; multiplying by division in one, two, or three directions of space; cells—spherical, cylindrical, comma-shaped, spiral, filamentous; or united into filamentous, flat, or cubical aggregates; filamentous aggregates often surrounded by a common sheath; plasma—homogeneous, without a morphologically differentiated nucleus; reproducing by simple fission; in some species resting bodies in the form of endospores and conidia; cells in certain species motile and possessing flagella.

The class of SCHIZOMYCETES is divided into the following six orders:—

1. EUBACTERIALES (true bacteria).—Undifferentiated simple forms; spherical, rod-shaped, or spiral; no true branching; some motile due to flagella; non-flexuous; multiplying by transverse fission; some produce endospores; no conidia.

2. ACTINOMYCETALES.—Rod-shaped or filamentous "mould-like" organisms with a tendency to branch and produce mycelium (e g. *Actinomyces*); often showing "club-shaped" formations; without spores but producing conidia in some species; mostly Gram-positive; non-motile; some are parasites of animals and plants; aerobic but some anaerobic; growth slow.

3. CHLAMYDOBACTERIALES.—Including the "iron bacteria," which are "plant-like" sheathed organisms, the sheath containing iron oxide.

4. THIOBACTERIALES.—Including the "sulphur bacteria," which are unsheathed cells and produce sulphur granules or bacteriopurpurin in their protoplasm.

5. MYXOBACTERIALES.—Including the myxobacteria; organisms exhibiting a pseudo-plasmodial and a resistant cyst-forming phase; "slime-mould" like in nature.

6. SPIROCHÆTALES.—"Protozoon-like" in certain characters; usually relatively slender, flexuous spirals; multiplying apparently in some species by longitudinal fission, though transverse fission is the characteristic mode of division as in other bacteria.

It may be noted that in this scheme the first order constitutes the *lower bacteria* as above described, whilst the next four orders make up the *higher bacteria*. The organisms pathogenic to the human subject belong to the orders 1, 2, and 6.

The families and genera of importance in medical bacteri-

ology are briefly described in the following summary; type-species are also given, with page-references to these in the general text of the book.

ORDER: EUBACTERIALES

Families:

NITROBACTERIACEÆ.—Motile or non-motile, non-sporing bacillary forms characterised by their capacity to utilise simple inorganic compounds. The nitrifying and nitrogen-fixing bacteria of soil belong to this family. None of these organisms is pathogenic.

COCCACEÆ.—Non-sporing and for the most part non-motile spherical or globose forms. These embrace the *cocci* described above.

SPIRILLACEÆ.—Definitely curved, rod-shaped, or non-flexuous spiral forms. Many species are motile and have terminal flagella. These include the *Vibrios* and *Spirilla* as already described.

BACTERIACEÆ.—Motile or non-motile, straight, rod-shaped forms which are non-sporing. They generally require complex organic substances for food and energy.

BACILLACEÆ.—Like the Bacteriaceæ but characterised by the formation of endospores.

FAMILY: COCCACEÆ

The more important Genera are:

Diplococcus.—Gram-positive cocci uniformly arranged in pairs, *e.g.* the pneumococcus (p. 326).

Streptococcus.—Gram-positive cocci arranged in chains (p. 286).

Neisseria.—Gram-negative cocci in pairs, *e.g.* the meningococcus (p. 350).

Staphylococcus.—Cocci which tend to be grouped in irregular masses or clusters; generally Gram-positive (p. 278).

Gaffkia.—Gram-positive cocci which tend to be arranged in tetrads, e.g. *Micrococcus tetragenus* (p. 318).

Micrococcus.—Saprophytic or facultatively parasitic cocci which occur in plates or irregular masses; generally Gram-positive; some species produce yellow or orange pigment; the type species is *Micrococcus luteus*.

Sarcina.—Cocci arranged in packets of eight, division occurring in three planes at right angles.

FAMILY: SPIRILLACEÆ

Genera:

Vibrio.—Curved, rigid, rod-shaped forms occurring singly or in pairs end to end or in spiral chains; motile, with usually a single terminal flagellum; generally Gram-negative. Many species are saprophytic water organisms, some are parasitic, e.g. *Vibrio choleræ* (p. 560).

Spirillum.—Described as rigid rod-shaped forms of varying thickness, length, and pitch of spiral, forming either long screws or portions of a turn; generally motile, with multiple terminal flagella; mostly saprophytes, *e.g.* in water.

Family : Bacteriaceæ

The more important Genera are :

Pseudomonas.—Saprophytic or parasitic Gram-negative bacillary forms which can be cultivated aerobically and produce a green or blue-green soluble pigment; e.g. *Bacillus pyocyaneus* (p. 318).

Lactobacillus.—Gram-positive, non-motile, bacillary forms which are frequently long and slender; form abundant acid from sugars; some are thermophilic; tend to be micro-aerophilic; occur in milk and milk products; e.g. *Bacillus acidophilus* (p. 901).

Bacteroides.—Motile or non-motile, non-sporing, anaerobic Gram-positive bacilli, which commonly occur as inhabitants of the alimentary tract of man and animals; e.g. *Bacillus bifidus* (p. 901).

Listerella.—Short, motile, Gram-positive, rod-shaped forms occurring singly or in pairs; strictly aerobic. *Bacterium monocytogenes*, an organism found in an infection of rabbits characterised by a pronounced monocytosis (Murray, Webb, and Swann), is assigned to this genus (p. 403).

Pasteurella.—Parasitic, Gram-negative, bacillary forms tending to exhibit bipolar staining, usually non-motile; aerobes; associated with hæmorrhagic septicæmia in animals and plague in man; e g. *Bacillus pestis* (p. 597).

Klebsiella.—Short, Gram-negative, encapsulated bacilli; aerobic; ferment various carbohydrates; occur as parasites in the upper respiratory passages and may be associated with inflammatory lesions in this region; *e.g.* pneumobacillus (p. 348).

Hæmophilus.—Parasitic, non-motile, Gram-negative bacillary forms which tend to be relatively minute; grow aerobically and only in the presence of certain factors derivable from blood; e.g. *Bacillus influenzæ* (p. 579).

Dialister.—Minute, non-motile, Gram-negative parasitic bacilli which grow only under anaerobic conditions and in media containing fresh tissue or serum; occur in the upper respiratory passages; e.g. *Bacterium pneumosintes* (p. 588).

Escherichia.—Gram-negative, aerobic bacilli commonly present in intestinal tract of mammalian animals; some are motile and possess peritrichous flagella; fermentation of carbohydrates, particularly glucose and lactose, with acid or acid and gas formation is a characteristic feature, but gelatin is not generally liquefied; e.g. *Bacillus coli* (p. 507).

Aerobacter.—This genus has been differentiated by American systematists from *Escherichia*, to which it is closely related, by its production of acetyl-methyl-carbinol from glucose as indicated by the occurrence of the Voges-Proskauer reaction (p. 510). Such organisms may also occur in the intestinal tract. The genus includes *Bacillus lactis aerogenes* (p. 514). These organisms are non-motile, tend to produce an abundant viscid growth and can be differentiated from the typical *B. coli* by their fermentation of inosite, their capacity to utilise salts of organic acids (*e.g.* sodium citrate) and in some cases by the Voges-Proskauer reaction (referred to above). It is doubtful if this supposed genus can be separated biologically from that designated *Klebsiella*, and its separation from *Escherichia* is also somewhat artificial.

Proteus.—Saprophytic and parasitic Gram-negative pleomorphic bacilli which are motile and possess peritrichous flagella; grow aerobically and tend to produce a "spreading" growth on culture medium; ferment glucose but not lactose; liquefy gelatin; e.g. *Bacillus proteus* (p. 320).

Salmonella.—Parasitic, Gram-negative, aerobic, usually motile bacilli associated with inflammatory conditions of the intestine of man and animals; ferment various carbohydrates but not lactose or saccharose; do not liquefy gelatin. This genus is exemplified by the paratyphoid bacilli and *Bacillus enteritidis* of Gaertner (p. 534).

Eberthella.—Similar to *Salmonella*, but gas formation is absent in the fermentation of carbohydrates. The typhoid bacillus is the type species (p. 517).

Shigella.—Similar to *Eberthella* but invariably non-motile; *e.g.* the dysentery bacilli (p. 547).

Brucella.—Small, Gram-negative, non-motile bacillary or cocco-bacillary forms which are pathogenic to man and animals; some species require for primary growth in culture medium an increased carbon dioxide concentration; do not ferment carbohydrates; gelatin is not liquefied; e.g. *Bacillus abortus* (p. 620).

Alcaligenes.—Motile or non-motile Gram-negative bacilli which may occur as commensals in the alimentary tract of man and animals; do not ferment carbohydrates and do not liquefy gelatin; e.g. *Bacillus fæcalis alkaligenes* (p. 554).

Family: Bacillaceæ

Genera:

Bacillus.—Gram-positive, sporing, bacillary forms which tend to occur in chains and form rhizoid colonies; mostly saprophytes; generally liquefy gelatin; e.g. *Bacillus subtilis* (p. 505). The anthrax bacillus (p. 486) belongs to this genus.

Clostridium.—Like *Bacillus* but anaerobic or micro-aerophilic; many species are parasitic and pathogenic; e.g. *Bacillus tetani* (p. 626).

ORDER: ACTINOMYCETALES

Families:

Actinomycetaceæ.—Characterised by their filamentous elements which are often branched and interlaced forming a mycelium; conidia are sometimes produced; some species are parasitic.

Mycobacteriaceæ.—Rod-shaped forms which are only occasionally branched or filamentous; conidia are not produced; the protoplasm often stains unevenly.

Family: Actinomycetaceæ

Genera:

Leptothricia.—Typical filamentous forms which are unbranched; these filaments may fragment into shorter rod-shaped elements; conidia not produced; Gram-positive when filaments are young; parasites; e.g. *Leptothrix buccalis* (p. 482).

Actinomyces.—Typical filamentous forms which branch and form mycelium, but may segment into shorter bacillary or coccal elements ; sometimes parasitic and pathogenic ; when growing in tissues the colonies may show radial, club-shaped structures at their periphery ; some species are anaerobic or micro-aerophilic ; e.g. *Actinomyces* (p. 466).

Erysipelothrix.—Gram-positive, rod-shaped forms with tendency to develop filaments which may branch ; micro-aerophilic ; usually parasitic ; *e.g.* Bacillus of swine erysipelas (p. 482).

Family : Mycobacteriaceæ

The important Genera are :

Mycobacterium.—Non-motile, rod-shaped forms which stain with difficulty but when stained are acid-fast ; club-shaped forms are sometimes observed and occasionally also elongated and branched elements ; aerobic ; growth on culture medium is often slow ; several species are pathogenic ; *e.g.* tubercle bacillus (p. 407).

Corynebacterium.—Parasitic, Gram-positive, non-motile, rod-shaped organisms, with a tendency to club-shaped forms and may show branching in old cultures ; stain unevenly ; non-acid-fast ; aerobic ; *e.g.* diphtheria bacillus (p. 374).

Fusiformis.—Usually elongated and fusiform organisms which stain unevenly ; non-motile ; non-branching ; anaerobic or micro-aerophilic ; e.g. *Bacillus fusiformis* (p. 662).

Actinobacillus.—Gram-negative, rod-shaped forms, sometimes filamentous and tending to branch ; aerobic, produce acid but no gas from carbohydrates ; pathogenic to animals and man ; e.g. *Actinobacillus lignieresi* (p. 477), and *Bacillus mallei* (p. 458).

ORDER : SPIROCHÆTALES

The important genera are :

Borrelia.—These are parasitic spirochætes with coils of relatively long wave-length and usually three to seven in number. They are fairly refractile under the microscope and are readily stained with the usual aniline dye solutions. The relapsing fever spirochætes (p. 682) are examples of this genus.

Treponema.—Parasitic forms with undulating or rigid spirilliform body, the coils being of relatively short wave-length, and often showing great regularity. They are feebly refractile and not readily stained by the usual dye solutions. The spirochæte of syphilis is classified in this genus (p. 667).

It should be noted that the spirochætes comprising these two genera, *Borrelia* and *Treponema* are frequently classified together and designated by the generic name *Treponema.*

Leptospira.—Parasitic and saprophytic forms consisting of sharply twisted filaments and with one or both ends " hooked " or recurved ; feebly refractile and difficult to stain by the usual dye solutions ; the coils are fine and numerous (as seen by dark-ground illumination) ; differ from the other groups in their ability to survive in distilled water; e.g. *Leptospira icterohæmorrhagiæ* of infectious jaundice (p. 692).

Variation and Dissociation

Recent bacteriological literature contains many references to the subject of variation among the bacteria, and the remarkable diversity of biological types met with in certain groups necessitates careful consideration of this question. A distinction must, of course, be drawn between the "fluctuating" variations in biological characters of different strains round the average or standard characteristics of a particular species, and the development of new types differing in some character from the original and retaining the new feature in successive generations.

Pleomorphism is a common feature of bacterial morphology, and it is well recognised how the microscopic appearances of bacteria may vary somewhat with their environment. Even under the same conditions, individuals tend to differ from one another though presenting an average or standard appearance. Thus a bacillus may vary in length from an almost coccal form to an elongated filament. Variation from the typical morphology is specially marked in culture, and in some cases more so after prolonged artificial culture. Thus capsule formation may be lost; motile organisms may become non-flagellate. Some workers have attached considerable significance to morphological variations and claim to have demonstrated markedly diverse morphological types in single species, and have thus postulated a life-cycle among the bacteria. A filter-passing stage has also been described. The question of such phases has already been discussed (p. 7). It is generally agreed, however, that among the pathogenic bacteria, species are fairly uniform in their morphology.

What may be called physiological variations are of frequent occurrence. In a bacterial growth the individuals are not uniform in character, but vary in their properties, powers of resistance, etc., and when we consider that many generations may occur in twenty-four hours, it is evident how readily variations may come into prominence under different environmental conditions. Variations in the biochemical or fermentative actions of bacteria are often met with and have been especially studied in the coli-typhoid group, as will be afterwards described. Thus new chemical properties may be acquired, and this may often occur spontaneously in culture, but is met with especially under two conditions, namely, (*a*) when the organisms are grown for a long time on a medium containing a particular constituent, *e.g.* a sugar, or (*b*) when a substance somewhat

inimical to growth is added to the medium—in both cases the organisms become adapted to their surroundings. The occurrence of such variations is sometimes indicated by the appearance of papillæ on certain of the colonies, and when an indicator such as neutral red is used along with the fermentable substance, these are of different colour, *e.g.* red papillæ on white colonies. In many of the examples given the variants revert to the original type after subculture, but in certain instances they are permanent. The latter occurrence was observed first by Neisser and by Massini in the case of "*B. coli mutabilis*," and similar phenomena were afterwards observed and fully described by Penfold. Twort, by growing the typhoid bacillus for a long time in lactose medium, found that it acquired the property of fermenting this sugar; but this is a rare result and others have failed to effect the change. The evidence points to such variants being the result of adaptation rather than mere selection.

Attention has of late been directed to differences in the appearance of colonies occurring in pure cultures of many organisms, both when obtained directly from the body and also from cultures grown in artificial media for some time; and variations have been found to arise even in cultures originally obtained from a single bacterium. The term "microbic dissociation" has been proposed by De Kruif, Hadley, and others for such cultural transformations. Certain of the differences tend to persist for long periods through repeated sub-cultures. Baerthlein studied the production of these variations and found that they are specially liable to develop in old cultures in fluid media; another method which facilitates their production is the growth of the culture in the presence of homologous antiserum. The action of a bacteriophage is also highly effective in bringing about dissociation. As Arkwight originally pointed out in the case of *B. dysenteriæ* (Shiga), two forms of colonies may be obtained on plating—a smooth ("S") and a rough ("R") type; the former resembles the normal colony character as usually described. The rough variant, while it yields the characteristic cultural reactions of Shiga's bacillus as regards fermentation of sugars and non-production of indole, may differ in other properties. Thus it may agglutinate spontaneously in 0·85 per cent. NaCl solution, although forming a stable suspension in weaker concentrations. A very important character in which the two types may show differences concerns their immunological properties; this is dealt with fully in Chapter VI. Here it may be said that serologically the two forms may be distinct—an

antiserum to the S form agglutinates not only the homologous smooth culture but also all smooth cultures of the organism; on the other hand, the S antiserum has little action on any of the R variants, whereas these are agglutinated by an anti-R serum, which contains no agglutinins for the S form. Similarly S and R forms have been obtained from many groups of organisms, including the coli-typhoid group, the *Pasteurella* group, and also the streptococci and pneumococcus. In addition to these types intermediate forms occur. There are frequently differences also in microscopic appearances of the individual organisms constituting the two types of colony; thus, among capsulated organisms, *e.g.* the pneumococcus, the variant may be devoid of a capsule. The different types of colony may correspond with marked differences in virulence, as De Kruif found in the case of a *Pasteurella* organism. Here the S type was highly virulent and the R type non-virulent. Similar observations have been made for other organisms, but there is no constant correspondence between roughness and lack of virulence. Arkwright showed also for *B. paratyphosus A* that vaccines of the R type, which was the less virulent, did not confer protection against infection with the S type. Once organisms have passed into the R condition, it appears that they are not readily susceptible to conversion again into the S type by procedures such as frequent subculture or animal passage; but in order to obtain variants which are persistent in character, repeated plating and selection of colonies for subculture may be required. In the *Salmonella* group again, variants exist which are characterised solely by the possession of "group" and "specific" antigens respectively; these differences are not permanent, and they are not associated with any distinctions in appearance of colonies. A peculiar type of dissociation has been described by Hadley, for example, in the typhoid and dysentery groups, consisting in the development of minute coccoid or rod-shaped forms which can pass through an earthenware filter. These variants produce very small colonies ("G" colonies) on culture medium. They slowly revert to the original form. The nature and significance of this type of dissociation require further elucidation.

As regards the significance of microbic dissociation, the changes brought about in a special environment have been interpreted as evidence of the life cycle of the organisms. One serious difficulty in connection with this view seems to be the stability of the R form. In the case of the pathogenic bacteria at least, the association of alterations in colony form with changes

in virulence suggests strongly that here one is dealing with a phenomenon akin to that resulting from the interaction between host and parasite. The conversion in the body of the virulent S type into the non-virulent R form might be regarded as evidence of a protective mechanism in the host. Though filter-passing variants of some of the ordinary bacteria have been described, this work requires extension and confirmation.

In general it may be said that the variations described among the pathogenic bacteria in no way interfere with the accepted biological classification. The species recognised retain certain basic characteristics; any variations observed represent subsidiary differences from the standard type.

The whole subject of variation and dissociation is too complex for extended consideration in this introductory chapter; but will be developed in relation to individual bacterial species, and will also be dealt with in its immunological aspects.

Bacteriophage

It has long been known that bacteria in culture are apt to undergo a process of autolysis, becoming disintegrated and sometimes disappearing; such changes are much more rapid and pronounced in some species than in others. Within recent years, however, it has come to be recognised that rapid lysis may be set up by certain external agents or stimuli, and that the lytic process is transmissible to other cultures by the products of the lysis. Twort (1915), in working with certain micrococci obtained from vaccine lymph, observed the appearance of glassy and transparent patches in cultures on agar, which increased at the expense of the ordinary bacterial growth. He found that a similar lytic change could be transmitted to fresh growths by inoculation from a glassy patch, and that in this way the change could be continued for an indefinite period of time. Further, he showed that the lytic agent was present in bacterium-free filtrates which had been passed through the finest porcelain filters. He discussed the various possibilities in explanation of the phenomenon, including that of its being due to an ultra-microscopic virus; but considered that a definite conclusion was not warranted. A similar phenomenon was described somewhat later (1917) by d'Herelle, first in the case of a dysentery bacillus, and was made the subject of a long series of important researches. He formed the opinion that the lysis was due to an ultra-microscopic virus and, in fact, represented the result of a parasitic infection of the bacteria

by the virus. He applied the term "bacteriophage" to the supposed virus, and this term has come into common use; it is often used in the abbreviated form of "phage." Various phages have now been obtained which act on many different organisms, including the Gram-negative intestinal commensals and pathogens, *V. choleræ, B. pestis,* staphylococci, streptococci, *B. diphtheriæ,* etc.

When an agar slope culture is treated with bacteriophage, a clear band develops along the line of inoculation, and this may involve the whole culture, the surface of the medium becoming clear of growth (Fig. 3). Sometimes colonies develop on the clear surface after a time, and these are formed from resistant organisms which have escaped the lytic action. When

FIG. 3.—Growth of *B. paratyphosus B.* on agar: the dark lettering PHAGE, is due to lysis of the organisms where the culture has been inoculated with the bacteriophage.

a young broth culture, turbid in appearance, is inoculated with bacteriophage, it gradually becomes clear as the lysis goes on and ultimately all trace of turbidity may disappear. But if the broth is inoculated with the organisms and at the same time a very small amount of phage is introduced, multiplication of the bacteria occurs unchecked for four or five hours and then clearing rapidly takes place. Fluid cultures are seldom completely sterilised by the phage; turbidity develops again later, owing to the growth of resistant organisms. If a well-developed young culture of an organism in broth be inoculated, and then some of it be spread over an agar plate the film of growth comes to be beset with small circular clear areas or "plaques," which represent points of action of the lytic principle or, according to d'Herelle's view, "colonies of the bacteriophage" (Fig. 4). When the bacterial colonies on a plate are of some size, those affected by the lytic process may be partly liquefied and present a "nibbled" appearance. As regards the changes produced in

the bacteria by the action of phage, it has been found in the case of *B. coli* that the cells swell up and then suddenly undergo solution, whereas *B. megatherium*, for example, shows no preliminary enlargement and the final stage of lysis lasts up to ten minutes. With both organisms, after the process is complete, irregular debris persists. Lysis of susceptible bacteria under the influence of phage does not occur in gelatin cultures, although the phage may undergo increase, neither does it occur in the presence of tissues, as in tissue cultures; also, the addition of serum to a preparation of phage inhibits its action. Again, phage does not lyse organisms suspended in saline, nor does it increase under these conditions, but both occur if some broth is added. Phage may multiply in cultures in synthetic media. Traces of calcium are necessary both for the lytic action and propagation of the bacteriophage. Propagation of the bacteriophage, so far as is known, takes places only in the substance of *living and multiplying* bacteria; and no one has been able to show any increase of it in any medium not containing the bacteria. On the other hand, killed bacteria may fix the phage, and so also exceptionally may living organisms which are insensitive to its action.

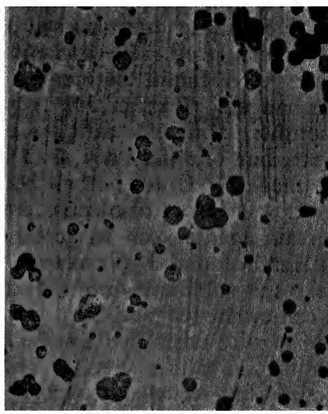


FIG. 4.—Plate culture of staphylococcus with "colonies" of bacteriophage; the latter appear as dark spots on the pale background of growth.

Method of Obtaining.—Samples of bacteriophage have most frequently been obtained from the alimentary tract of the human subject and animals, either in a healthy or diseased condition. They have also been got from organisms cultivated from lesions in the tissues, and, further, they have occasionally appeared to develop in ordinary cultures in the laboratory. The usual method of obtaining a bacteriophage, say from the intestine, is to make a culture in broth, then filter and test for the presence of the lytic property by inoculating a given culture of an organism with the filtrate. After a bacteriophage has been obtained, its action on a given organism can frequently

be increased by passing from culture to culture; a certain level of activity is then maintained by further passages. Occasionally, however, it dies out spontaneously; and this occurs also if a minute dose of phage is added to great excess of the susceptible culture. It is noteworthy that certain cultures, although consisting entirely of organisms insensitive to phage, nevertheless yield a lytic agent for other organisms; such cultures are termed *lysogenic.*

Powers of Resistance.—In comparison with most unicellular organisms, bacteriophage has comparatively high powers of resistance to various agencies. For example, a lysed culture may contain the lytic principle after several years, as is shown by inoculating from it to a fresh culture of the microbe originally lysed. Fæces kept in sealed tubes for over a year have been found to contain the bacteriophage practically unchanged. Further, it may withstand drying for several months. Different samples show variation in resistance to heat, but one may say that in some instances a temperature of about 75° C. for half an hour is necessary to ensure destruction. It shows also considerable resistance to treatment with certain agents such as acetone, alcohol, ether, chloroform, etc. D'Herelle found that it was not destroyed after being kept in 1:200 corrosive sublimate or in 1:100 carbolic acid for three days; after a somewhat longer period destruction followed. Some phages are readily destroyed by the action of strong solutions of urea or neutral salts of quinine and a few by trypsin. A staphylococcus phage was destroyed by the photodynamic action of methylene blue in the presence of oyxgen, but when fixed to living organisms either of homologous or heterologous species, it was protected. The presence of citrate in the medium prevents the multiplication of some phages but not of others. Kabéshima showed that when a lysed culture was precipitated by acetone, a fine powder was thrown down and that this contained the lytic agent unchanged for about six weeks. It may also be mentioned that bacteriophage persists for a long period in strong solutions of glycerol, in this respect corresponding to the filterable viruses. According to Baker and Nanavutty, the susceptibility of phage to ultra-violet rays is of the same order as that of bacteria, whereas ferments and antibodies are much less sensitive. In the case of a lysogenic strain of the sporing bacillus, *B. megatherium*, den Dooren de Jong found that the property of producing phage persisted after the spores had been heated for five minutes at 100° C., whereas the phage itself was destroyed at 70° C. in the same time. Similarly, in

the case of a spored culture of *B. subtilis* artificially inoculated with the corresponding phage, after the spores had been boiled and allowed to germinate the resulting culture contained phage (Cowles).

Nature.—Regarding the true nature of the transmissible lytic agent there has been, and still is, much controversy. Various theories have been, put forward, but these essentially centre around two, namely, (*a*) that the agent is a living ultra-microscopic virus, and (*b*) that it is a non-living ferment supplied by the bacteria themselves. It is generally agreed that the actual lytic process depends upon an enzyme and that fixation of the phage by the bacteria is a necessary preliminary. The question at issue is whether it is produced by a definite virus, or whether it is formed by the bacteria themselves. With regard to the former, one has to note that the supposed virus is beyond the limits of microscopic vision, and as yet it has not been observed directly, still less its multiplication by division. Furthermore, such a virus has never been cultivated in any medium apart from the susceptible bacteria in the living state; and lastly, the facts with regard to resistance to various agents do not appear to correspond with those of any known living organism. In these circumstances it does not seem justifiable to assume a living organism unless there is no possibility of other explanation. Those who regard the agent as non-living consider that it is an autolytic enzyme transmissible from bacterium to bacterium. According to Bordet and Ciuca, the bacteria become modified in some way, so that an inherent tendency to autolytic processes is exaggerated. This modification is transmitted to the descendants of the bacteria, and thus the lytic process is described as being hereditarily transmissible. The autolysin diffuses in the medium and sets up similar autolysis in other bacteria. Such a view involves the possibility of an enzyme giving rise to, or setting free, more enzyme of similar nature, as the lytic agent undoubtedly increases in amount. Bordet has pointed out as an analogy that thrombin in bringing about coagulation of the blood also leads to increase of itself.

Bacteriophages obtained from different sources present considerable differences in respect to the organisms on which they exert a lytic action. A phage active against the bacillus of dysentery, for instance, has, as a rule, a restricted action on members of the same group, especially on varieties of *B. coli*; as a rule it has little or no action on the organisms of other groups. It has been found, however, that when a phage is added

repeatedly to cultures of a resistant organism, *e.g.* by propagating it in a mixture with the susceptible bacterium, lytic properties towards the former may be developed. Further, it has been found that when lytic action for one organism is acquired it may be lost for another. D'Herelle regards the change in the lytic properties as a process of adaptation and as evidence that the phage is a living organism. The phenomenon seems, however, explicable also on the enzyme theory, since if one enzyme can lead to the activation of another, a more active enzyme may be set free and its effects will become increasingly manifest. In any case, a phenomenon allied to adaptation is an observed fact. Again, Bail has shown that when a filtrate of material which naturally contains phage, such as fæces, is allowed to act on a susceptible culture in dilutions which produce discrete plaques, the latter may not all be of the same size. When phage from a large plaque is propagated further in cultures, it continues to produce similar plaques ; in the same way, phage derived from small plaques reproduces these. Another constant feature of the plaques is the nature of their margins. Further, if a strain of organisms resistant to each type of phage is developed in the manner described above, it is found that such resistance is specific—that is, the strain which has been rendered resistant to the one type remains susceptible to the other. A culture resistant to several different phages can be obtained by subjecting it to these successively. Also, when an organism has developed resistance as the result of treatment with a particular specimen of phage, it may in consequence become susceptible to other phages which were without action on it in its original state.

Bordet found that from a specimen of phage which acted both on the smooth and the rough components of a culture of *B. coli*, a special phage was obtained by the following method : it was added to an excess of culture, the latter not being sufficient, however, to prevent altogether the reproduction of the phage. The result was that the phage obtained under these conditions was no longer able, even in high concentrations, to lyse completely the organisms of the original culture. This " weak " phage still acted on the smooth organisms of the culture, but was almost without effect on the rough variant ; hence, when it was added to the culture, the latter variant developed. However, by repeated passages in cultures of the rough type, the phage acquired activity toward the latter also. If now the " strong " phage was allowed to act on the smooth organisms, these could be rendered resistant to it ; such organisms, however, retained their sensitiveness to the weak phage.

The relation of bacteriophage to bacterial variation has been mentioned ; as will be seen, the latter is associated with changes

in antigenic constituents of the organisms (p. 39). While the action of phage on the S type leads to the appearance of R forms, the reverse may also occur, though rarely. But acquired resistance to phage may not be accompanied by any change in the antigenic characters of an organism detectable by means of agglutinating antisera (Burnet). Again, the S and R forms of a given bacterium differ in their susceptibility to phage action; as a rule the latter are acted on by a wider variety of phages than the former. In this respect also there is considerable parallelism between the distribution of a common somatic antigen in different bacterial species and their sensitiveness to a given phage. This has been confirmed by the finding that a complex labile polysaccharide extracted from Salmonella bacilli has the property of combining with the corresponding phage and preventing its action on the susceptible bacilli (Gough and Burnet).

The particulate nature of phage has been demonstrated by physical methods. Thus, Schlesinger has caused the particles to sediment by powerful centrifugalising and has estimated their size from the energy required to bring them down. Elford and Andrewes by the use of filters composed of collodion membranes of graded porosity, have shown that different phages which act on the same organism may vary greatly in size from 8–12 $m\mu$ to 50–75 $m\mu$. On the other hand, a particular phage does not undergo alteration in the size of its particles as a result of growth in different species of susceptible organisms or after purification. The phages with the smaller particles are those which produce the larger plaques, owing to their greater diffusibility. It has been found that under optimum conditions one plaque results from a single particle of phage and Burnet, by counting the number of plaques obtained when a young culture containing phage is plated from time to time, has concluded that each bacterium gives rise to a number of particles of phage.

Bacteriophage possesses antigenic properties which are distinct from those of the organisms on which it acts; thus phage-containing material when injected into animals develops antibodies which inhibit the lytic action. Bordet and Ciuca found that this effect is produced by antiserum which has been heated at 60° C.; in this respect its action differs from that of bactericidal sera. The antiserum also agglutinates the phage particles. By means of such antisera, Watanabe confirmed the difference between different phages which act on the same culture of organisms, since the antilytic effect is exerted only against the homologous phage.

A concentrated purified preparation of *B. coli* bacteriophage investigated by Schlesinger when injected into rabbits produced an antiserum which inhibited the phage, but had no agglutinating action on the corresponding organisms. This preparation of phage manifested practically no respiratory metabolism in relatively large amounts either by itself or with dead organisms belonging to the susceptible culture, or in a sub-lytic dose along with a living culture. It possessed no ferment property except phosphatase; but other phosphatases derived from tissues were devoid of phage action. Accordingly, taking into consideration all the facts, it does not seem justifiable at present to express a definite view as to the nature of bacteriophage.

CHAPTER II

METHODS OF CULTIVATION OF BACTERIA

Introductory.—When bacteria have penetrated into the deep tissues and the circulation during life, it is the rule to find only a single species present. But genuine mixed infections are met with, and invasion of organisms frequently occurs *ante* or *post mortem*. Also, when an infective disease involves a mucous membrane or the skin, or when an open lesion has developed, a variety of organisms is generally found. In order to discover which of a mixture of bacteria is responsible for a specific disease, it is necessary to study each separately. Accordingly, to obtain pure cultures is a chief requisite of bacteriological research. Since bacteria are practically omnipresent, means are needed for destroying all extraneous organisms which may be in the food media, in the vessels containing these media, and on all instruments which are to come in contact with the cultures. The technique of this destructive process is called *sterilisation*. Further, the growth of bacteria in other than their natural surroundings involves the preparation of *sterile artificial food media*; when such media have been prepared we have still to consider the technique of the *separation of micro-organisms from mixtures of these*, and *the maintaining of pure cultures when the latter have been obtained*. Different methods are necessary according as *aerobes* or *anaerobes* are dealt with.

Methods of Sterilisation

Underlying the methods commonly used is the general principle that all bacteria are destroyed by heat. The temperature necessary varies with different bacteria, and the vehicle of heat is also of great importance. The two vehicles employed are hot air and hot water or saturated steam. The former is usually referred to as "dry heat," the latter as "moist heat." As showing the different effects of the two vehicles, Koch found, for instance, that the spores of *Bacillus anthracis*, which were killed by moist heat at 100° C. within one hour, required three hours' dry heat at 140° C. to effect death. Both

forms of heat may be applied at different temperatures—in the case of moist heat above 100° C., a pressure higher than that of the atmosphere must, of course, be developed.

A. *Sterilisation by Dry Heat*

A (1). **Red Heat and Flaming.**—Red heat is used for the sterilisation of platinum wires. A dull red heat is used for cauteries, or the points of forceps. Similarly, small objects may be sterilised by "flaming," *i.e.* passing them repeatedly through the flame; this method is used for cover-slips, slides, the mouth of culture tubes, cotton-wool stoppers, paper, etc.; but care must be taken not to melt or char the objects. Needles and scalpels may be sterilised by keeping them in alcohol, which is burned off before use.

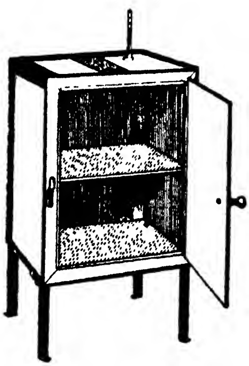


FIG. 5.—Hot-air steriliser (simple form).

A (2). **Sterilisation by Dry Heat in a Hot-Air Oven.**—The oven (Fig. 5) consists of an outer and inner case of sheet iron. In the bottom of the outer there is a large hole. A Bunsen is lit beneath this, and thus plays on the bottom of the inner case, round all the sides of which the hot air rises and escapes through holes in the top of the outer case. A thermometer passes down into the interior of the chamber, half-way up which its bulb should be situated. It is found, as a matter of experience, that an exposure in such a chamber for one hour to a temperature of 170° C. is sufficient to kill all the organisms which usually pollute articles in a bacteriological laboratory, though circumstances might arise where this would be insufficient. Electrically heated ovens are now often used; by means of a regulator the temperature can be maintained at the desired height. This means of sterilisation is used for glass apparatus, *e.g.* flasks, test-tubes, Petri dishes, pipettes, and throat swabs. Cotton-wool plugs are not damaged by the temperatures employed and should be inserted before heating.[1] Such pieces of apparatus are thus obtained sterile and dry. It is

[1] The cotton-wool must be of a suitable quality. H. D. Wright has found that with plugs of non-absorbent wool or inferior grades of absorbent wool dry heat above 140° C. often leads to the formation of a film of material on the interior of the tubes, perhaps of fatty nature, which is inhibitory to the growth of organisms such as pneumococci.

advisable to have the glass vessels dry and to put them into the oven before heating it, and not to open the door after sterilisation till the temperature has fallen, since sudden heating or cooling is apt to cause glass to crack. The method is manifestly unsuitable for culture media which contain water.

B. *Sterilisation by Moist Heat*

B (1). By Boiling.—The boiling of a liquid for five minutes is sufficient to kill all non-spored organisms, but some spores may resist boiling for an hour and a half or even longer. This method is useful for sterilising distilled or tap water, and for tubes, syringes, instruments, etc. To minimise rusting of knives and steel instruments it is well to boil the water for some time before placing them in it. The form of steriliser commonly used is an enamel-ware fish-kettle pattern, which should be provided with a removable tray with a raised edge, to prevent articles from falling off.

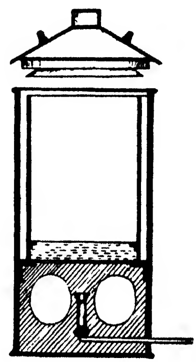


FIG. 6.—Koch's steam steriliser (simple form) in section.

B (2). By Steam at 100° C.—This is by far the most useful means of sterilisation for culture media. The apparatus ordinarily used is "Koch's steam steriliser" (Fig. 6). This in its simplest form consists of a tall metal cylinder on legs, provided with a lid, and covered externally by some bad conductor of heat, such as felt or asbestos. A perforated diaphragm is fitted in the interior at a little distance above the bottom, and there is a tap at the bottom by which water may be supplied or withdrawn. If water to the depth of several inches be placed in the interior and heat applied, it will quickly boil, and the steam streaming up will surround flasks, etc., standing on the diaphragm. Here no evaporation takes place from any medium, as it is surrounded during sterilisation by an atmosphere saturated with water vapour. It is convenient to have the cylinder tall enough to hold a litre flask with a funnel 7 inches in diameter standing in its neck. The funnel may be supported by passing its tube through a second perforated diaphragm placed in the upper part of the steam chamber. With such a "Koch" in the laboratory a hot-water filter is not needed. The Koch's steriliser may be heated by passing into

the water steam from a steam-heating system or by electricity. (If the Koch is attached to the hot water supply pipe by a ball-cock the water in it will be maintained automatically at a constant level.) One and a half hour's steaming will usually sterilise any watery fluid, but in the case of media containing gelatin such an exposure is not practicable, as, with long boiling, gelatin tends to lose its physical property of solidification. The method adopted in this case is to *steam for twenty minutes on each of three succeeding days.*

This is a modification of what is known as "Tyndall's intermittent sterilisation." The fundamental principle of this method is that all bacteria in a non-spored form are killed by the temperature of boiling water, while if in a spored form they may not be killed. Thus by the sterilisation on the first day all the non-spored forms are destroyed —the spores remaining alive. During the twenty-four hours which intervene before the next heating, these spores, being in a favourable medium, are likely to assume the non-spored form. The next heating kills these. In case any may still not have changed their spored form, the process is repeated on a third day. Experience shows that usually the medium can now be kept indefinitely in a sterile condition.

Steam at 100° C. is therefore available for the sterilisation of all ordinary media. In using the Koch's steriliser, especially when a large bulk is to be sterilised, it is best to put the medium in while the apparatus is cold, in order to make certain that the whole of the mass reaches the temperature of 100° C. The period of exposure is reckoned from the time boiling commences in the water in the steriliser. At any rate, allowance must always be made for the time required to raise the temperature of the medium to that of the steam surrounding it. It is advisable to cover cotton-wool stoppers of flasks and tubes with several layers of parchment or "Kraft" paper to prevent excessive wetting.

B (3). Sterilisation by Saturated Steam at High Pressure.—This is the most rapid means of sterilisation. It is effected in an autoclave (Fig. 7). This is a gun-metal cylinder surrounded by a cylindrical sheet-iron case; its top or end, according as it is placed vertically or horizontally, is fastened down with screws and nuts and rendered airtight by a washer. There are a safety-valve, tap, and pressure-gauge. As in Koch's steriliser, the contents are supported on a perforated diaphragm. The source of heat is a large Bunsen beneath, or the autoclave may be heated by electricity or from a steam heating system. The following pressures in pounds per square inch (which include the 15 lb. due to ordinary atmospheric pressure) correspond

to the temperatures shown: 18 lb.=105·8° C.: 20 lb.=109° C.: 25 lb.=116° C.: 30 lb.=121·5° C.: 35 lb.=126° C. The desired temperature is maintained by adjusting the safety-valve so as to blow off at the corresponding pressure. One exposure of media to a temperature of 115°–120° C. for a quarter of an hour is sufficient to kill all organisms or spores.

Certain precautions are necessary in using the autoclave. Except where the heat is derived from circulating steam, care must be taken to ensure the presence of sufficient water, so that there is a residuum when steam is fully up, otherwise the steam is superheated, and the pressure on the gauge does not indicate the temperature correctly. The procedure in using the autoclave is as follows: Insert the materials to be sterilised; then fix down the lid and apply the heat. Leave the tap open until a steady jet of steam escapes from it, since if all the air is not expelled, a mixture of air and steam being present, the pressure shown by the gauge will not accurately indicate the temperature. Then close the tap and reckon the time of sterilization from the point when the gauge shows the required pressure. After ceasing to apply heat, it is necessary to let the apparatus cool well below 100° C. before opening it or allowing steam to blow off, otherwise there will be a sudden development of steam when the pressure is removed, and fluid media will be blown out of the flasks. In order to prevent cotton-wool stoppers from becoming wet they should be covered with several layers of parchment or "Kraft" paper. When medium is to be sterilised in bulk, however, time must be allowed for attaining the required temperature; the same is the case with masses of fabric, *e.g.* surgical dressings, which must be completely penetrated by the steam. The requisite information is got by placing recording thermometers in the centre of the material to be sterilised. Autoclaving is the best method of rendering infected material innocuous, such as old cultures, especially when spores are present. It should also be used for sterilising test tubes, etc., fitted with cotton-wool plugs, followed by drying at 120° C. (*see* footnote p. 50). Gelatin or media containing substances which are readily

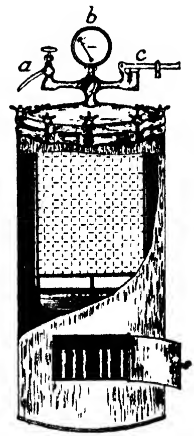


FIG. 7.—Autoclave.
a. Exhaust valve.
b. Gauge.
c. Safety valve.

decomposed, *e.g.* carbohydrates, must not be exposed to temperatures above 105° C., and are best sterilised by the intermittent method.

B (4). Sterilisation at Low Temperatures.—Many organisms in a non-spored form are killed by prolonged exposure to a temperature of 57° C. This fact has been taken advantage of for the sterilisation of blood serum or other materials containing protein, which will coagulate if exposed to a temperature above that point. Such a medium is sterilised on Tyndall's principle by exposing it for an hour at 57° C., for eight consecutive days, it being allowed to cool in the interval to the room temperature. This is best effected in a water-bath heated by means of gas or electricity and provided with a temperature regulator.

Serum sterilisers are constructed in which the test-tubes are placed in the sloped position, and in which inspissation (*vide* p. 67) can afterwards be performed at a higher temperature. Bacterial cultures which are intended for use as vaccines are sterilised by heating in a water-bath, the temperature of which should be as low as is compatible with killing the organisms, *e.g.* for one hour at 60° C. in most cases.

C. *Sterilisation by Chemicals*

Volatile antiseptics such as chloroform may be used to preserve serum which is to be kept for preparing media: 5 c.c. chloroform are added to each 100 c.c. of serum, and the mixture, in a stoppered bottle, is shaken well at frequent intervals for several days, and is then stored at room temperature. Before use all undissolved chloroform is removed from the serum; the latter may then be exposed in a thin layer at 57° C., but this is not essential when Löffler's medium is to be made, as the remaining chloroform volatilises in the process of slow heating required for coagulating and sterilising the serum.

D. *Sterilisation by Filtration*

In some cases fluids, such as serum, are sterilised most effectively by filtration through filters whose pores are too small to let bacteria pass. Filtration is dealt with later (p. 98).

Maintenance of Sterility

It is essential to prevent recontamination of sterilised materials, *e.g.* from the dust in the air. For this reason, flasks, test-tubes, etc., are closed with firmly fitting stoppers of cotton wool which

are inserted before sterilising. When the contents have to be kept sterile for a considerable time, it is well also to cover the stoppers with sterile paper which is tied on round the neck of the vessel, or with indiarubber caps. McCartney has introduced screw-capped bottles as a substitute for plugged flasks and test tubes as receptacles for media.[1] Petri dishes and pipettes should be wrapped in paper before sterilising, and if they are to be stored they should be sterilised in a metal box with close-fitting lid which is sufficiently deep (3 in.) and whose top is flush with the rim of the body. Many articles, such as capillary pipettes, are conveniently sterilised and stored in wide glass "boiling" tubes plugged with cotton wool.

The Preparation of Ordinary Culture Media

The general principle to be observed in the artificial culture of bacteria is that the medium used should resemble that on which the bacterium grows naturally. In the case of pathogenic bacteria the medium therefore should resemble the fluids of the body. Thus blood serum is often used. Other media have been found which can support the life of most of the pathogenic bacteria. These consist of proteins or carbohydrates in a fluid, semi-solid, or solid form. It is an advantage to have a variety of media, since growth characters on particular media, non-growth on some and growth on others, etc., constitute specific differences which are valuable in the identification of bacteria. The most commonly used media have as their basis a watery extract of meat *plus* salt and "peptone" (broth or bouillon). Most bacteria growing in such a medium cause only a grey turbidity, scum, or sediment without characteristic appearances of aid in classification. But such a medium is valuable for investigating the soluble toxins of bacteria. A great advance resulted when Koch, by adding gelatin to broth, provided a transparent solid medium in which growth characteristics of particular bacteria become evident. Many organisms, however, grow best at a temperature at which this nutrient gelatin is fluid, and therefore another gelatinous substance called agar, which does not melt below 98° C., was substituted. The reaction of the medium is important, since most bacteria, in contrast to moulds, prefer a neutral or slightly alkaline reaction. It is to be noted that, in general, when preparing media the minimum amount of heating should be used, as prolonged exposure to high temperatures

[1] These are supplied by United Glass Bottle Manufacturers, Ltd., 40–43 Norfolk Street, Strand, London, W.C.2.

damages their nutritive properties. Recent investigations have shown that the value of nutrient media may be seriously impaired by certain metallic contaminations with the result that, while large inocula of organisms may grow, small ones fail. O'Meara and MacSween found in the case of staphylococcus that copper derived from utensils or from some specimens of peptone produced this effect. A curious fact is that the medium after heating does not become inhibitory until it has been kept for some time. The advantage of methods of preparing media in which the peptone is added directly to the minced meat seems to depend chiefly on the absorption of the copper by the meat particles. It is likely also that the above method of preparation avoids oxidation of the peptone (H. D. Wright). Also avoidance of filtration through excessive thicknesses of paper, cloth, etc., prevents absorption from the medium of hormones which promote the growth of delicate organisms (Lloyd and Cole).

Preparation of Broth

The flesh of the ox, calf, or horse is usually employed. Horseflesh has the advantage of being cheaper and containing less fat than the others; though generally quite suitable, it has the disadvantage for certain purposes of containing a larger proportion of fermentable sugar. The flesh must be freed from fat and finely minced. To 500 grams of mince add 1000 c.c. distilled water, 10 grams of peptone [1] and 5 grams of sodium chloride. Mix well and heat for 20 minutes at 68° C., stirring at intervals. Shake well and steam in the Koch for 30 minutes; filter through paper [2] and adjust the reaction to pH 7·8–8·0 (p. 61). Steam again for 30 minutes and filter through paper. Check the reaction (pH 7·6–7·8) and add 0·15 per cent. glucose; tube and sterilise in the autoclave at 115° C. for 10 minutes.

The commercial meat extract "Lab-Lemco" added to water in the proportion of 10 grams to 1 litre forms a substitute for the meat extract in the preparation described above.

Glucose Broth. — To the above broth after the final filtration add 1 or 2 per cent. of glucose. Tube and sterilise as above.

Glycerol Broth.—Add 1½ to 6 per cent. of glycerol (sp. grav. 1·25) to digest broth or to ordinary broth *after filtration*. This medium is especially used for growing the tubercle bacillus when the products of the growth of the latter are required. The lower proportion of glycerol is generally advisable.

Digest Bases for Media

It has been found that by partially digesting minced meat or casein with trypsin a very suitable substitute for meat extract and

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[1] Commercial peptone is a mixture of albumoses and peptones. B.D.H. bacteriological peptone is suitable for most purposes. Other reliable brands are Difco and Witte's.

[2] Balston's "Whatman No. 1," or "Postlip thick grey" filter paper is suitable.

peptone results. Contact of these media with copper utensils must be avoided at all stages.

Horse Heart Digest (Short Method of Preparation—modified from Douglas).—Horse heart (freed from fat and fibrous tissue) is minced finely and is mixed thoroughly with water in the proportion of 500 grams mince to 1 litre water. The mixture is rendered strongly alkaline to litmus paper with 4 per cent. NaOH solution, and is allowed to stand at room temperature for half an hour, when the reaction is again tested, more alkali being added should the mixture be acid; it is now heated in the Koch's steriliser at 80° C. for half an hour. Then it is cooled to 40° C. and 5 c.c. "liquor trypsinæ co."[1] and 10 c.c. chloroform are added; the mixture is well shaken several times and is allowed to stand at room temperature for fifteen minutes before being placed in the incubator. After incubating at 37° C. for twenty-four hours sufficient 10 per cent. HCl is added to render the reaction acid to litmus paper. The mixture is placed in a flask, which should be about two-thirds full, in the cold Koch and the burner is lighted, so that the temperature is raised slowly and the chloroform evaporates completely; finally it is boiled in the Koch for one and a half hours and thereafter is cooled and filtered. The filtrate is made up to 1 litre with distilled water. This solution constitutes horse heart digest. It may be sterilised at 115° C. for fifteen minutes in plugged flasks and stored for the preparation of broth, agar, etc.

Heart Digest Broth is prepared by adding to the above heart digest 0·5 per cent. NaCl and sufficient 4 per cent. caustic soda solution to render the reaction distinctly alkaline to litmus paper.

Long Digest is prepared as above, but the mixture with trypsin is kept in a loosely stoppered bottle at 37° C. for twenty-one days; after the first ten days' incubation a further quantity of 5 c.c. liquor trypsinæ co. is added. From the commencement the reaction must be tested daily with litmus paper and 4 per cent. NaOH solution added as required to keep the mixture alkaline, since an acid reaction destroys the trypsin and so prevents progress of the digestion. Media prepared with Long Digest are specially useful for obtaining primary cultures of delicate organisms, *e.g.* gonococcus, meningococcus, and *B. influenzæ*, and also for growing the tubercle bacillus. They are also valuable when abundant cultures are required, *e.g.* for the preparation of vaccines.

Long Digest Broth is prepared as in the case of Heart Digest Broth above.

Casein Digest.—Mix 200 grams "laitproto No. 6 for bacteriological purposes"[2] into a smooth paste and make up to 1000 c.c. with cold water; then add slowly a boiling solution of 20 grams anhydrous sodium carbonate in 500 c.c. water, stirring vigorously: pour the mixture into a flask and heat in the Koch at 100° C. for ten minutes. Transfer the mixture to a large bottle and wash out the flask with 500 c.c. water, which is also transferred to the bottle. Cool to 40° C., add 50 c.c. liquor trypsinæ co. and 15 c.c. chloro-

[1] This preparation of trypsin is made by Allen & Hanburys, 7 Vere Street, Cavendish Square, London, W. Hartley found that owing to its containing glycerol, it is unsuitable for media for certain purposes, *e.g.* for obtaining diphtheria toxin, when trypsin prepared from pig's pancreas should be used, as recommended by Cole and Onslow (p. 58).

[2] Obtainable from Casein Ltd., Culvert Works, Battersea, London, S.W.

form ; shake well and keep the bottle loosely stoppered at 37° C. for five days (shaking daily) : then add another 50 c.c. liquor trypsinæ co. and keep the mixture at 37° C. for ten days further. Finally shake well to break up tyrosine masses and pour the whole into a large flask ; add 400 c.c. N /1 HCl, steam for thirty minutes and filter ; then add to the filtrate 120 c.c. N /1 NaOH, finally adjust the reaction to slight alkalinity as tested with litmus paper, and sterilise.

Casein Digest Broth is prepared by adding 1000 c.c. of the above digest to 2000 c.c. 0·5 per cent. sodium chloride solution, adjusting the reaction and sterilising.

Media prepared with digests as a basis yield much more luxuriant growths of most organisms than do those prepared from ordinary meat extract with peptone ; but the latter are preferable for keeping stock cultures, as they maintain the viability of the bacteria for a longer time.

Hartley's Broth

This is a medium very suitable for the production of diphtheria toxin. One hundred and fifty grams of minced horse muscle are mixed with 250 c.c. tap water and heated to 80° C. in a steamer : 250 c.c. of 0·8 per cent. sodium carbonate solution (the anhydrous salt) are then added, and the mixture cooled to 45° C., after which 5 c.c. of chloroform and 5 c.c. of pancreatic extract [1] (Cole and Onslow) are added (the amount of pancreatic extract required varies with different preparations). The mixture is incubated at 37° C. for six hours, the vessel being shaken at frequent intervals. Forty c.c. of normal hydrochloric acid are then added, and the mixture heated in a steamer for half an hour, then cooled and filtered. The reaction of the filtrate is adjusted to pH 8, and the medium distributed into containers. For the sterilisation of small quantities (100 c.c. medium in half-litre bottles) free steam is passed through the autoclave for one hour, then the pressure is raised slowly to 10 lb. and the steam turned off. For larger quantities (1 litre of medium in double Winchester quart bottles) the same method of sterilisation is adopted, except that the pressure is maintained at 10 lb. for half an hour.

Peptone Water

A simple solution of peptone constitutes a suitable culture medium for many bacteria. The peptone in the proportion of 1 to 2 per

[1] The fresh pancreas of a pig is at once, on removal from the newly killed carcase, placed in 50 per cent. spirit and brought to the laboratory ; there it is freed from fat as far as possible, and weighed. It is minced finely and three times its weight of distilled water and its own weight of absolute alcohol are added. Shake the mixture well in a large bottle and allow it to stand for three days at room temperature, shaking the bottle occasionally. Strain through muslin and filter through a large folded filter paper. The filtrate, which comes through very slowly, is measured and treated with 1 c.c. of concentrated hydrochloric acid for every litre. This causes the appearance of a cloudy precipitate which settles in a few days and can be filtered off. The fluid keeps for an indefinite period if stoppered, without any additional antiseptic. If desired at once, the extract may be used before adding hydrochloric acid, the function of which is to retard the slow auto-destruction of the trypsin.

cent., along with 0·5 per cent. NaCl, is dissolved in distilled water by heating. The fluid is then filtered, placed in tubes, and sterilised. The reaction is usually distinctly alkaline, which condition is suitable generally. For special purposes the reaction may be standardised. Peptone water is much used for testing the formation of indole by bacteria; and by the addition of one of the sugars to it the fermentative powers of an organism may be tested (p. 77), an indicator being added to show any change in reaction.[1]

Cooked Meat Medium

A medium containing minced heart muscle was introduced by Robertson especially for the cultivation of anaerobes. The following is Lepper and Martin's modification of this medium: 500 grams of fresh bullock's heart are minced, placed in 500 c.c. of boiling N/20 caustic soda and allowed to simmer for twenty minutes, by the end of which time the neutralisation of the lactic acid will be ensured and the pH of the liquor should be about 7·5. The liquid is drained off on a muslin filter, and while still hot the minced meat is pressed in a cloth and allowed to dry partially by being spread on a cloth or filter-paper. In this condition it can be introduced into test-tubes without soiling them. Enough should be placed in each to occupy about 5 cm. of the tube, and 1 per cent. peptone broth, adjusted to pH 7·5 added until the liquid stands 1 cm. above the meat. The tubes must be kept in a bath of boiling water for half an hour to drive off the entangled air; then they are autoclaved at 120° C. for twenty minutes The inoculum should be introduced towards the bottom of the tube in contact with the pink meat. A similar medium may be prepared with brain.

1. **Gelatin Media.**—These are simply broth, with gelatin added as a solidifying body. Highly characteristic growths of organisms take place on gelatin media, which are liquefied by certain species. Gelatin media have, however, the disadvantage of not being suitable when growth is to take place at temperatures above 24° C., as the gelatin melts. For higher temperatures agar must be employed.

1 (*a*). **Ordinary Gelatin** :—

Meat extract . .	1000 c.c.	or digest broth 1000 c.c.
Sodium chloride . .	5 grams	
Peptone . . .	10 ,,	
Gelatin . .	100–150 ,,	

(The "gold label" gelatin of Coignet et Cie, Paris, is the best.) The sheets of gelatin are rolled up and added with the other constituents to the extract; the mixture is allowed to stand at room temperature for fifteen minutes and is then thoroughly melted in the "Koch" (about half-an-hour's heating suffices). The fluid medium is then well agitated and is rendered slightly alkaline. At this stage it is practically always necessary to clear the medium. This is done by first cooling to 50° C., then adding the white of one egg, slightly beaten up, to each 1000 c.c. of medium, along with a

[1] It is essential to test the peptone for the absence of sugar and indole *See also* p. 81.

few pieces of cotton or glass wool, and then boiling in the Koch for one and a half hours. The resulting coagulum carries down any particles which cause turbidity. Thereafter the medium is filtered through Chardin's [1] paper, care being taken that the contents of the flask are not shaken up. As the medium must not be allowed to solidify during the process, it must be kept warm. This is effected by putting the flask and funnel into a tall Koch's steriliser, in which case the funnel must be supported on a tripod or diaphragm, as there is great danger of the neck of the flask breaking if it has to support the funnel and its contents. The filtration may also be carried out in a funnel with water-jacket which is heated. In either case it is advisable to lay a glass plate over the filter funnel to prevent evaporation or condensation water dropping into the medium. A litre flask of the finished product ought to be quite transparent. The flask containing it is then plugged with cotton wool and sterilised, best by method B (2) p. 51. Too much boiling, or heating at too high a temperature, as has been said, causes a gelatin medium to lose its property of solidification. The exact percentage of gelatin used in its preparation depends on the temperature at which growth is to take place. Its firmness is its most valuable characteristic, and to maintain this in hot summer weather 15 parts per 100 are necessary. But if the gelatin be too stiff, it will split when punctured by the platinum wire used in inoculating it with a bacterial growth ; 15 per cent. gelatin melts at about 24° C. For ordinary use in British laboratories 10–12 per cent. gelatin is a sufficient strength.

1 (*b*). **Glucose Gelatin.**—The constituents and mode of preparation are the same as 1 (*a*), with the addition of 1 to 2 per cent. of glucose before sterilisation. This medium is used for growing anaerobic organisms at the ordinary temperatures.

2. **Agar Media (French, "gélose").**—These have the advantage over gelatin that at blood temperature (38° C.), at which most pathogenic organisms grow best, they are solid. Agar is a carbohydrate derived from the stems of various seaweeds growing in the Chinese seas, commercially classed together as "Ceylon Moss." For bacteriological purposes the dried stems of the seaweed may be used, but there is in the market a purified product in the form of fibre, shreds or flakes,[2] which is preferable. Agar medium does not melt until about 98° C. and when fluid remains so on cooling down to about 40° C. Though quite clear when fluid, on solidifying it always becomes slightly opaque. Further, growths upon it are often less characteristic than those on gelatin. It is not liquefied by any of the pathogenic bacteria.

2 (*a*). **"Ordinary" Agar.**—A generally suitable agar medium is prepared as follows (modified from Huntoon) : 25 grams agar

[1] The original French *Papier Chardin* in unfolded sheets should be used.
[2] "B.D.H." and "Bacto" agar are reliable. Of the latter 1·5 per cent. is equivalent in stiffening properties to 2·5 per cent. of most other preparations.

fibre in fine fragments (or 15 grams Bacto agar) are dissolved in 1 litre water by heating in the autoclave; after cooling to 50° C., 500 grams minced ox or horse heart muscle are added, and the mixture is boiled in an open pan for fifteen minutes with constant stirring. Then 10 grams peptone are added slowly and 5 grams sodium chloride, gentle boiling being continued for about five minutes until the ingredients are dissolved. Solid particles are removed by straining through coarse iron wire gauze into a tall glass cylinder; the reaction of the fluid is brought to pH 7·8, and it is steamed in the Koch for thirty minutes; then the flame is turned out, and the whole is allowed to stand overnight so that cooling occurs slowly. Next morning the solid medium is removed from the cylinder, the deposit is cut off, and the rest is melted in the Koch, then stored in a plugged flask or tubed. Sterilisation is effected either in the autoclave for 15 minutes at 115° C. or in the Koch on three successive days.

The following method is also satisfactory

Digest broth . . .	1000 c.c.
Agar	15–25 grams.

Add the agar in fine fragments to the other ingredients and allow to stand for a quarter of an hour. Then boil gently in a "Koch" for one to two hours, or autoclave at 115° C. for thirty minutes, till the agar is thoroughly melted. Clear as in the case of gelatin. Render slightly alkaline with sodium hydroxide solution, and if necessary make up to original volume with distilled water, and filter through Chardin's paper as in the case of gelatin. If a slight degree of turbidity may be tolerated, it is sufficient to filter through a felt bag or jelly strainer. Sterilise as usual.

2 (*b*). **Glycerol Agar**.—To 2 (*a*) before sterilisation add 1½–6 per cent. of glycerol and sterilise as above. This is used especially for growing the tubercle bacillus.

2 (*c*). **Glucose Agar**.—Prepare as in 2 (*a*), but add before the final sterilisation 10 per cent. sterile solution of glucose sufficient to make the concentration 1–2 per cent. This medium is used for the culture of anaerobic organisms at temperatures above the melting-point of gelatin.

2 (*d*). **Peptone-Water Agar** is prepared by dissolving the usual amount of agar in peptone water (p. 58) and sterilising.

Semi-Solid Agar

A semi-solid medium has been found useful for various purposes, *e.g.* increasing the motility of flagellated bacteria and obtaining growths of organisms which do not flourish in a fluid medium or on ordinary solid agar. It is prepared by adding to any of the fluid bases 0·3–0·5 per cent. of agar and proceeding as usual. It may be used as a thin layer in plates or in deep tubes.

Adjustment of the Reaction of Media.—The adjustment of the reaction of bacteriological media is a matter of great complexity. The method usually adopted with a medium whose basis is meat extract, which is ordinarily slightly acid, is to add

saturated sodium carbonate or sodium hydroxide solution till the reaction is slightly but distinctly alkaline to red litmus paper and no longer affects blue litmus paper. The occurrence of an amphoteric reaction—*i.e.* one where red litmus is turned blue, and blue, red—is thus avoided. The test paper must be immersed in the liquid—on no account is the sampling to be done by transferring drops to the paper by means of a glass rod. The disadvantages of this method are that ordinary litmus is not a delicate indicator and, further, no standardisation of the proper tint to be aimed at is possible.

Estimation of True Acidity or Hydrogen-Ion Concentration.—The true acidity of any fluid depends on the number of free or dissociated hydrogen ions which it contains, or, as it is ordinarily expressed, on the *hydrogen-ion concentration.* The greater the number of free hydrogen ions the greater is the acidity; the stronger an acid is, the larger is the proportion of its hydrogen in the free or ionised state. The standard solution (in relation to which the concentration is expressed) is 1 gram of free hydrogen ions in a litre of water. Such a standard would correspond with a normal solution of strong acid, say hydrochloric acid (36·5 grams HCl in a litre of water, H = 1 gram), provided that the hydrogen was completely dissociated. As a matter of fact, however, not all the hydrogen is in the form of free ions. In a similar way the hydrogen-ion concentration of decinormal hydrochloric acid is slightly less than a tenth of the standard, but when the solution of acid is very dilute nearly all the hydrogen ions are free. In the case of a weak acid, however, the hydrogen-ion concentration is often only a fraction of that of hydrochloric acid. Accordingly, no definite relationship exists between normal, decinormal, etc. solutions of acids and their true acidity.

True alkalinity, in a corresponding fashion, depends on hydroxyl-ion concentration, and a fluid is neutral when it contains an equal number of free hydrogen and free hydroxyl ions. Pure distilled water is, of course, such a neutral fluid; only a very small proportion of its hydrogen is in the ionised state, the hydrogen-ion concentration being $\frac{1}{10^7}$, that is, 10^{-7}, and its hydroxyl-ion concentration is, of course, the same. According to the system now ordinarily used, the hydrogen-ion concentration, represented as pH, is expressed by the logarithm of the concentration with its sign changed. Thus, in the case of a neutral fluid, *e.g.* distilled water, the hydrogen-ion concentration is 10^{-7}; its logarithm is -7, and therefore its pH $= 7$. Similarly,

if we used an exponent for the hydroxyl-ion concentration, the pOH of a neutral fluid would be 7 also. Further, the product of hydrogen-ion concentration and hydroxyl-ion concentration is a constant, and pH + pOH always = 14. The reaction of a fluid is ordinarily expressed as pH, but the true alkalinity (pOH) can always be obtained as it = 14 − pH. Owing to the conventional change in the sign, the pH increases as the acidity diminishes, and any pH greater than 7 means an alkaline reaction ; that is, the fluid contains more hydroxyl ions than hydrogen ions. For example, the blood serum and the tissue fluids, which are slightly alkaline, have a pH of approximately 7·5 ; and, as a rule, this is the optimum reaction of media for the growth of pathogenic bacteria.

It is necessary that the worker should understand that there is an important difference between the acidity of a solution as estimated by the amount of standard solution of alkali necessary to neutralise it and the true acidity, *i.e.* between the " titration acidity " and the hydrogen-ion concentration. For example, N/10 acetic acid requires as much alkali to neutralise it as N/10 hydrochloric acid, when tested in the usual way by an indicator ; whereas the hydrochloric acid contains several times the number of free hydrogen ions which acetic acid does—that is, is several times as strong an acid.

Another important subject is that of *buffers* in solution, that is, substances which diminish the amount of free ions when they are added to a solution. When, for instance, a certain amount of hydrochloric acid is added to a solution of sodium acetate, sodium chloride is formed with the setting free of acetic acid and water, and the acetic acid is less ionised than the hydrochloric acid ; thus the mixture contains fewer free hydrogen ions than the acid added. Amongst such substances which act as buffers are various salts, like carbonates, phosphates, citrates, etc., amino-acids and proteins ; all these act in the way indicated. Buffer substances are abundantly present in ordinary bacterial media, and thus, even when the true neutral point is known, the desired pH cannot be obtained merely by the proportional addition of a standard solution of acid or alkali.

Estimation of pH.—The pH of a solution can be directly determined only by means of an electrical method, involving the use of the hydrogen electrode. Such a method is impracticable for ordinary bacteriological laboratories, and a colorimetric method which gives satisfactory results is ordinarily used instead. This depends on the fact that when indicators are changed in colour by the reaction of a medium, there are two

points in pH between which the change takes place and that a particular tint given by an indicator corresponds with a definite pH. The procedure is thus to determine what amount of alkali is necessary to bring a given amount of the medium to a tint corresponding with the desired pH ; alkali is then added to the whole volume of the medium in proportionate amount.

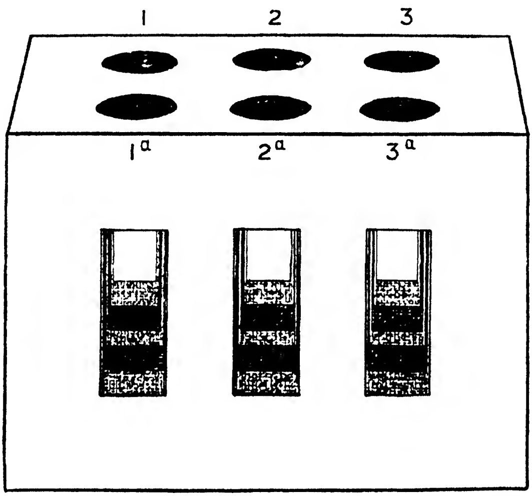

FIG. 8.—Comparator used in estimation of hydrogen-ion concentration.

Solutions of graded pH can be suitably prepared by mixing M/15 di-sodium phosphate (Na_2HPO_4) and M/15 mono-potassium phosphate (KH_2PO_4) in varying proportions. Such solutions with a suitable indicator added, say phenol red, can be purchased.[1] They are supplied in hard white glass tubes—the so-called " cordite " tubes—of uniform diameter and thickness of glass. And similar tubes are used in making comparative estimations. For example, a series of tubes of pH 6·8—8·4 (with differences of 0·2) with phenol red show variations in tint from pale yellow to crimson. We have, in the first place, to ascertain how much alkali has to be added to a given quantity of medium—5 c.c., in order to bring it to the colour of the desired reaction, say pH 7·5. In such a case it is convenient to use two tubes with pH 7·4 and 7·6 respectively, and to bring the tint of the medium to a point intermediate between the two.

[1] Colour standards are not permanent and should be checked at least once a year.

A comparator rack to hold two rows of tubes is used and is so arranged that each pair of tubes (front and back row) can be superimposed and examined by light transmitted through them. The scheme of such a comparator is shown in Fig. 8. In 1 and 3, two standard tubes with pH 7·4 and 7·6 are placed; each contains phenol red to show the corresponding tint. In front of each in 1*a* and 3*a* a tube of medium is placed; in 2 a tube of distilled water, and in 2*a* a tube containing 5 c.c. of medium and 0·5 c.c. of a 0·01 per cent. solution of phenol red in 30 per cent. alcohol.[1] Thus the light transmitted through each pair of tubes passes through the same constituents, namely, (*a*) a tube of medium, (*b*) a tube of water or colourless solution, and (*c*) a solution of phenol red; the difference being that in the case of the standard tubes the indicator is in the phosphate solution, whereas in the tubes to be tested the indicator is in the medium. The alkali to be added is in the form of N/20 sodium hydrate solution, with phenol red added. This is prepared by taking 500 c.c. of N/10 NaOH, adding 45·5 c.c. of 0·02 per cent. phenol red, and making up to 1000 c.c. with distilled water. A micro-burette is necessary to deliver and measure the solution of alkali. For this purpose a 1 c.c. pipette graduated to 0·01 c.c. may be used; a fine pipette is attached to the lower end by a piece of rubber tubing with a pinchcock on the rubber to control delivery.[2]

Method.—Before standardising, it is convenient to bring the reaction of the medium to about pH 7 by the addition of sodium hydrate, for example to a reaction which just gives a fine pink with coralline (rosolic acid) paper. Of this medium place 5 c.c. in a cordite tube and add to it 0·5 c.c. of 0·01 per cent. solution of phenol red. Note the tint as seen in the comparator with a tube of water superimposed, and then gradually run in the N/20 NaOH solution till a tint intermediate between the tints of the two standard tubes is obtained. Repeat the process and take the mean of the two observations. Let the number of c.c. of N/20 NaOH solution $=X$; then $X \div 20 =$ the number of c.c. of a normal NaOH solution, and $\frac{X}{20} \times \frac{1000}{5} =$ the number of c.c. of normal NaOH necessary to bring a litre of medium to the required reaction, namely, pH 7·5

Instead of the series of standard tubes described above, a Lovibond Comparator[3] may be used, in which a measured quantity of the indicator is added to the medium and the colour is matched by looking through standard-coloured glasses behind which a similar tube of the medium (without indicator) is placed.

The description given applies to a fluid medium, such as broth.

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[1] Made by diluting a 0·02 per cent. solution in 60 per cent. alcohol with an equal volume of distilled water.

[2] British Drug Houses, London, supply a "Universal Indicator" mixture.

[3] Supplied by British Drug Houses.

It is best to sterilise the medium before standardisation; and after the reaction has been adjusted, subsequent sterilisation should be carried out at 100° C. Even in this case heating tends to raise somewhat the hydrogen-ion concentration, and a tube of the final product should be tested by adding the indicator. Gelatin may be treated in a similar way to that described, the medium being first liquefied and kept at a temperature of about 30° C.

In the case of agar it is troublesome to carry out the method as directed, owing to the temperature necessary to have the medium liquefied. The procedure usually followed is to bring the broth to the required reaction and then to add the agar and liquefy at 100° C. The agar should be previously brought to a fine state of division and well washed in water; if this is done the agar has usually only a trifling effect on the reaction. Here, again, a test of the reaction of the final product should always be made. In carrying out all these procedures, practice is necessary for the attainment of satisfactory results. For ordinary purposes Eyre's method as described below is satisfactory.

Eyre's Method of adjusting the reaction by titration is applicable to any of the media ordinarily employed. The reaction of a medium may be conveniently expressed by the sign + or − to indicate acid or alkaline respectively, and a number to indicate the number of cubic centimetres of normal alkaline or acid solution necessary to make a litre of the medium neutral to phenol-phthalein. Thus, for example, "reaction $= -15$," will mean that the medium is alkaline, and requires 15 c.c. of normal HCl to make a litre neutral. When a medium such as broth reacts neutral to litmus, its reaction to phenol-phthalein is acid; now, as litmus was introduced by Koch, and as nearly all bacterial research was at first done with media tested by litmus, it is difficult to say exactly what precise degree of alkalinity was originally determined to be the optimum for bacterial growth. It is probable that when a medium has been rendered neutral to phenol-phthalein by the addition of NaOH, the optimum degree is generally attained by the addition of from 10 to 15 c.c. of normal HCl per litre, *i.e.* the optimum reaction is from $+10$ to $+15$. According to Fuller, the optimum reaction for bacterial growth, as a rule, lies about midway between the neutral point indicated by phenol-phthalein and the neutral point indicated by litmus.

Procedure.—The medium with all its constituents dissolved is filtered and then heated for about forty-five minutes in the steamer, the maximum acidity being reached after this time. Of the warm medium measure 25 c.c. into a conical flask and rinse out the measure into the flask with a small amount of boiling distilled water, add 0·5 c.c. of 0·5 per cent. phenol-phthalein solution in 50 per cent. alcohol. Run in decinormal soda till neutral point is reached, indicated by the first trace of pink colour, the mixture being kept hot. To recognise the first tint of pink in the yellow broth, take two samples of the medium, adding the indicator to one only; then run the soda into these from separate burettes; for each few drops run into the medium containing the indicator the same amount

is run into the other. Thus the recognition of the first permanent change in tint will be at once recognised by comparing the two samples. Repeat process thrice, and take the mean; this divided by 10 will give the amount (x) of *normal* soda required to neutralise 25 c.c. of medium; then $40x$ = amount necessary to neutralise a litre; and $40x - 10$ = amount of normal soda necessary to give a litre its optimum reaction. Then measure the amount of medium to be dealt with, and add the requisite amount of N/1 or 10N caustic soda solution. When the acid or alkali has been added the reaction of the medium must be again taken before sterilisation.

Special Culture Media

Very many different media have been used either where special difficulty is experienced in getting an organism to grow, or where some special growth characteristic is to be studied. It is impossible to do more than give the chief of these.

Serum and Blood Media

Inspissated Blood Serum.—Koch introduced this medium for the cultivation of the tubercle bacillus, and in order to obtain it in a comparatively clear state, adopted the method of inspissation at 65° C. after sterilising by the intermittent method at low temperature—B (4) (p. 54). "Inspissation" is an initial stage of coagulation, and is effected by keeping the serum at 65° C. till it stiffens. This temperature is just below the coagulation point of the serum. The more slowly inspissation is performed the clearer will be the serum. The apparatus used for the purpose is one of the various forms of serum inspissator, the temperature being controlled by a regulator. The procedure is somewhat tedious, and for all ordinary purposes opaque coagulated serum, sterilised by the usual methods, can be substituted.

Coagulated Serum.—A sufficient quantity of serum is placed in a series of sterile test-tubes; these are then placed in a sloped tray, and heated in the Koch at 80° C. for an hour. If the process of heating is carried out too quickly, bubbles of gas are apt to form and to tear up the surface of the medium. This can be avoided if the serum is solidified high up in the Koch. Sterilisation is effected by heating the medium similarly at 80° C. for two hours on each of two successive days. Special ovens, which serve both for inspissation and sterilisation, may be used. The great advantage of this medium is that the serum need not be obtained with special aseptic precautions.

Method of collecting Blood for obtaining Serum.—Blood from a horse, ox, or sheep, obtained at a slaughterhouse, is suitable for most purposes. After the neck vessels have been cut and the blood has been flowing for some time (the first blood is liable to be grossly contaminated), the stream from the carotid artery is allowed to flow directly into a sterile, large, wide-mouthed stoppered bottle, which, when full, is at once closed and returned, without much shaking, to the laboratory. The coagulum is then separated from the glass with a strong sterile wire and the bottle is allowed to stand overnight, preferably in the ice-chest. The serum is then

pipetted off. If there is any admixture with red blood corpuscles, the latter can be removed by centrifuging. With care sterile serum can be obtained. When it is desired to use the uncoagulated serum as a culture medium it should be heated as described under B (4), and finally should be tested for sterility after incubating for forty-eight hours at 37° C.

Blood which is to be defibrinated is collected in the same way, but glass beads are placed in the receiving bottle before it is sterilised and the bottle is partially filled. It is then stoppered and shaken continuously for five to ten minutes.

In order to obtain sterile specimens of blood from large animals a cannula is inserted into the jugular vein.[1] In the case of a small animal such as a rabbit, sterile blood is most readily obtained by heart-puncture. A wide needle is fitted by a short piece of india-rubber tubing to a 100 c.c. pipette, the mouthpiece of which has been plugged with cotton wool, the apparatus being sterilised. When the animal is anæsthetised, the chest-wall is cleansed and the needle is passed into the heart; blood is then sucked into the pipette (50 c.c. per kilo of body-weight can be obtained), and is defibrinated in a sterile flask with glass beads (see above).

Small quantities of sterile blood may be obtained from the marginal vein of the rabbit's ear by the following procedure. The area is shaved and cleansed with alcohol and ether; when the skin is dry, melted paraffin at a temperature of about 54° C. is smeared thinly with cotton wool on both sides of the ear round the vein, and when by compression at the base of the ear the vein has been rendered turgid, it is incised transversely in the middle of the paraffined area. Care is taken that the blood comes into contact only with the paraffined skin. For other details see p. 149.

Blood from the human subject is obtained by venepuncture (p. 165).

Löffler's Blood Serum.—This is the best medium for the growth of the *B. diphtheriæ*, and may be used for other organisms. It has the following composition: Three parts of calf's or lamb's blood serum are mixed with one part ordinary neutral peptone broth made from veal with 1 per cent. of glucose added to it. Though this is the original formula, it can be made from ox or sheep serum and ordinary beef broth without its qualities being markedly impaired. Sterilise as in the case of coagulated blood serum (p. 67). If the medium has to be stored the tubes should be filled with 0·25 per cent. glucose broth. The method of preparation is otherwise the same as that described for Coagulated Blood Serum.

Media enriched with Blood or Serum

Blood-Smeared Agar.—This medium was introduced by Pfeiffer for growing the influenza bacillus, and it has been used for the organisms which do not readily grow on the ordinary media, *e.g.* the gonococcus and the pneumococcus. Human blood or the blood of animals may be used. "Sloped tubes" (*vide* p. 83) of agar are employed (glycerol agar is not so suitable). Purify a finger first with 5 per cent. liq. cresol. sap., dry, and then wash with absolute

[1] Sterile horse blood may be obtained commercially from Burroughs Wellcome.

alcohol. Allow the alcohol to evaporate. Prick with a needle sterilised by heat, and, catching a drop of blood in the loop of a sterile platinum wire, smear it on the surface of the agar. Then keep the tube sloped for about ten minutes until the blood has coagulated. Cover the tubes with indiarubber caps, and incubate them for one or two days at 37° C. before use, to make certain that they are sterile. Agar poured out in a thin layer in a Petri dish may be smeared with blood in the same way and used for cultures.

Serum-Smeared Agar is prepared in a similar way by smearing the surface of the agar with blood serum, or by adding a few drops of serum to the tube and then allowing it to flow over the surface.

Blood Agar.—For many purposes (*e.g.* the growth of the whooping-cough bacillus, the bacillus of soft sore, the cultivation of trypanosomes and Leishmaniæ) the use of agar containing defibrinated blood, especially rabbit blood, is desirable. The blood may be obtained as has been described.

However the blood is obtained, after defibrination it is warmed to 45° C., and added to agar of the same temperature in the proportion of about one-third of blood and two-thirds of agar (for most purposes the addition of a smaller amount of blood, *e.g.* 5 per cent., to the agar is satisfactory). Such media must be incubated before use to ensure that bacteria have not gained access during preparation.

Blood Broth.—The addition to broth of 10 per cent. uncoagulated sterile blood, *e.g.* rabbit's, yields a fluid medium in which many delicate organisms will grow.

Boiled Blood Medium (" Chocolate Agar ") is suitable for obtaining cultures of *B. influenzæ* and other organisms, such as gonococcus, meningococcus, pneumococcus, which grow with difficulty on ordinary agar. To 5 c.c. melted heart digest agar medium, at 60° C. add 0·5 c.c. (about 10 drops) defibrinated blood—rabbit's blood is suitable. Heat the mixture by immersing the tube in boiling water for one minute (not longer) ; then allow to solidify in the sloped position. Boiled blood broth is prepared similarly.

Serum Agar.—Certain delicate organisms, *e.g.* gonococcus, require for their growth uncoagulated serum, which should preferably be freshly drawn. Suitable medium consists of agar, especially that prepared with a digest basis ; this is melted and cooled to 50° C., then 5–10 per cent. of serum is added and, after mixing, the medium is sloped or poured into Petri plates. Usually animal's serum, *e.g.* rabbit's, serves well Sterile ascitic or hydrocele fluid may be used instead of serum.

Bordet and Gengou's Medium for Bacillus of Whooping-Cough.—An extract of potato is first prepared by adding two parts of water containing 4 per cent. of glycerol to one part of potato chips ; the mixture is then boiled and the fluid is separated off ; 1 part is mixed with 3 parts 0·6 per cent. sodium chloride solution, and the reaction is made slightly alkaline (pH 7·5) ; then 4 per cent. agar is added, and the whole is sterilised in the autoclave. For preparing plates the medium is tubed in amounts of 7 c.c. To a tube of the melted medium at 50° C., 3–5 c.c. of sterile defibrinated rabbit's or human blood (obtained by the method described on p. 165), warmed to 40° C., are added and uniformly mixed. The mixture is then poured into Petri plates. This medium is also very suitable for the growth of the gonococcus, meningococcus, and influenza bacillus.

Blood-Potato Agar (Kirkpatrick).—This medium yields good growths of many delicate organisms, e.g. *B. influenzae*, *B. pertussis*, the tubercle bacillus, gonococcus, meningococcus, pneumococcus, etc. It consists of Bordet-Gengou potato extract 100 c.c., long digest broth (p. 57) 300 c.c., agar fibre 10 grams (or Bacto agar 8 grams). Dissolve the mixture in the autoclave at 115° C. (thirty minutes); then render slightly alkaline to litmus with 4 per cent. caustic soda solution. Fill into $6 \times \frac{5}{8}$ inch test-tubes (6 c.c. per tube) and sterilise in the autoclave at 115° C. for fifteen minutes; then cool to 56° C. and add to each tube 0·5–1 c.c. fresh sterile defibrinated rabbit blood, mix thoroughly, heat in the Koch for fifteen minutes and slope.

Blood-Alkali Agar (Dieudonné).—This medium, introduced for the culture of the cholera vibrio, for which purpose it has been found extremely suitable, has the property of inhibiting the growth of most of the intestinal bacteria; for example, the *B. coli* does not grow on it, or does so very slightly. A blood-alkali solution is prepared by adding equal parts of defibrinated ox blood and of normal caustic soda solution; the solution is then heated in the Koch until it ceases to smell of ammonia (thirty minutes); it keeps for two months. Of this solution three parts are added to seven parts of ordinary agar medium or digest agar rendered neutral to litmus, and the mixture is poured into plates which, after drying, should be kept at room temperature for twenty-four hours before they are inoculated.

Egg Media

Media containing either the yolk, or both the yolk and the white of egg, have been used for the culture of the tubercle bacillus by Dorset and others. Also, cultures on egg medium are useful for preserving the life of many organisms for long periods. The following will be found very suitable:

Egg Medium.—Break into a sterile bowl fresh eggs, which have been washed in soap and water and then dried, and beat with a sterile knife to mix the yolks and whites. Strain the mixture through sterile cheese cloth (several layers of the cloth are stretched over a filter funnel and sterilised in the Koch); and to every 75 c.c. of egg (about two eggs) add 25 c.c. of sterile twenty-one days' heart digest broth and 1 c.c. of 1 per cent. watery solution of crystal violet. Fill the medium into sterile test-tubes, avoiding the formation of air bells, and coagulate by heating in the Koch at 80° C. (the tubes should be placed high up in the steriliser, in a sloping position). Sterilise by heating in the Koch at 80° C. for two hours on three successive days. The medium keeps for several months provided drying does not occur. If the surface of the medium has become dry before the inoculation of a tube, two drops of sterilised water are placed on the surface. The inoculation material is well rubbed over the surface of the medium, the tubes are sealed with a few drops of melted paraffin wax on the top of the plug and are incubated in the sloped position. Crystal violet has the property of inhibiting various other organisms which may be present as contaminants. It may be advisable to omit the dye when the

medium is intended for cultures of the bovine type of the tubercle bacillus.

Glycerol Egg Medium is prepared as above by using heart digest broth to which has been added 6 per cent. of glycerol.

Alkaline Egg Medium.—Beat up the yolk of one egg with the whites of two, then add 6 c.c. N/1 NaOH, and finally add slowly 500 c.c. tap water. Heat the mixture very slowly to 95° C. and keep it at this temperature for not less than one hour, then filter through cotton wool and muslin, tube and autoclave at 115° C. for twenty minutes. When cool, one part of the fluid may be added to five parts of broth.

Potatoes as Culture Material

Large potatoes are selected ; the surface is cleansed by scrubbing in running water and cylinders are cut with an apple corer or large cork borer. Wash in running water for two hours, to remove excess of starch. Divide the cylinders by a diagonal cut and place each half thus obtained in a sterile test-tube, the broad end of the cylinder resting on wet cotton wool. Fill the tubes with sterile water and steam in the Koch for half an hour. Pour off the water and autoclave at 115° C. for twenty minutes. Potatoes ought not to be prepared long before being used, as the surface is apt to become dry and discoloured. It is well to take the reaction of the potato with litmus before sterilisation, as this varies ; normally in young potatoes it is weakly acid. If required, the acidity may be neutralised by filling the tubes with 0·7 per cent. sodium bicarbonate solution (instead of water) before steaming. Potatoes before being inoculated ought always to be incubated at 37° C. for a night, to make sure that the sterilisation has been successful.

Glycerol Potato Medium.—The method of preparation is the same as in ordinary potato medium, except that 6 per cent. glycerol is used in place of the sterile water for filling the tubes.

Löwenstein-Jensen Medium for the Cultivation of the Tubercle Bacillus

1. *Salt-starch solution.*—A solution of the following ingredients [1] is prepared by heating in distilled water :

Potassium dihydrogen phosphate .	0·4	per cent.
Magnesium sulphate . . .	0·04	,,
Magnesium citrate . . .	0·1	,,
Asparagine	0·6	,,
Glycerol	2·0	,,

600 c.c. of the solution are steamed in the Koch for two hours and allowed to cool overnight. Then 30 grams of potato starch [1] are added and the mixture heated in a water-bath with constant stirring for fifteen to twenty minutes, until a satisfactory paste has formed ; this is kept for one hour in the water-bath at 56° C., after which time it is ready for adding to the egg-fluid.

2. *Egg-fluid.*—Hens' eggs (which must be less than one week old) are thoroughly washed in a 5 per cent. solution of soft soap,

[1] The chemicals may be obtained from British Drug Houses and specified " A.R."

and then left in running water for one to two hours. The eggs are now broken into a sterile vessel (20–22 eggs yield 1000 c.c.) and the yolks and whites rendered homogeneous by mixing for ten minutes by hand or in a shaking machine, after which the mixture is filtered through sterile gauze, and to 1000 c.c. are added the 600 c.c. of salt-starch solution prepared as above.

3. To 1600 c.c. of the mixture of salt-starch and egg-fluid add 20 c.c. of a 2 per cent. aqueous solution of *malachite green* in distilled water, which has been incubated for one to two hours beforehand.

The medium is now tubed in 5 c.c. amounts in 1 oz. screw-capped bottles, the caps being tightly screwed on. The bottles are laid horizontally in the inspissator and are heated at 85° C. for half an hour. They are allowed to remain at 37° C. overnight, and are heated again on the following day at 75° C. for half an hour. In these bottles the medium will keep for many months; but slopes in test-tubes must be stored in the cold and used within a month. This medium can be strongly recommended, particularly for the human type.

Liver Extract Agar for the Isolation of Bacillus Abortus

Fresh ox liver is minced and ground to pulp in a mortar; 500 grams of this are mixed with 500 c c. of tap water, steamed for 90 minutes and then filtered through iron wire gauze (60 mesh). In 500 c.c. of the extract are incorporated 30 grams washed agar, 10 grams peptone, 5 grams sodium chloride, and 500 c.c. tap water are added. The pH is adjusted to 7·0 and filtration is effected through iron wire gauze. The medium is tubed and autoclaved. The final pH should be 6·6. If the material cultivated is likely to contain other organisms the addition of gentian violet (in a concentration of 1 : 200,000) facilitates isolation of *B. abortus*.

Milk as a Culture Medium

This is a convenient medium for observing the effects of bacterial growth, in coagulating the soluble albumin, and in fermenting the lactose. It is prepared as follows: Boil well-skimmed milk in the Koch for ten minutes; allow to cool and filter through Chardin's filter paper. Add 6 per cent. litmus solution (p. 79) and adjust the reaction. Fill into test-tubes and sterilise for twenty minutes on three successive days. This is *litmus milk*. The litmus may be omitted. The reaction of fresh milk is alkaline. If great accuracy is necessary, any required degree of reaction may be obtained by the titration method.

Selective Media

Numerous media have been devised for the purpose of isolating particular organisms from a mixture containing similar types. They depend upon several principles. In one form a substance, *e.g.* a sugar, is added which is fermented with the production of acid by certain organisms which are likely to be present but not by others; an indicator dye is also incor-

porated in the medium so that the colonies of the fermenting organisms have a different colour from the non-fermenters. In the case of the coli-typhoid group such media usually contain lactose which is fermented by the common types of coliform bacilli in fæces but not by the specific pathogens. The indicator may be litmus or, better, some other dyestuff which is highly coloured in the presence of acid but pale or almost colourless when the reaction is neutral or alkaline. Accordingly, when examining the resulting growth attention is paid to the pale colonies. Another class of selective media contains some substance which in the concentration used inhibits the contaminating organisms while permitting the pathogens to grow, *e.g.* suitable concentrations of brilliant green tend to suppress the ordinary coliform types while typhoid and paratyphoid bacilli multiply. The bile salts in MacConkey's medium are used similarly to inhibit cocci. Again, media containing compounds of tellurium allow enrichment of diphtheroids while inhibiting other classes of organisms found in the throat and nose. The following are further examples of selective antiseptics which may be added to agar media in order to inhibit certain organisms while permitting the growth of others. Crystal violet in a concentration of about 1 : 100,000 allows most Gram-negative bacilli to grow while killing Gram-positive organisms. Thallium acetate about 1 : 1000 inhibits *B. pyocyaneus* and *B. proteus* while permitting growth of staphylococci and streptococci. Similarly, copper sulphate 1 : 2000 prevents the spreading growth of *B. proteus* while permitting many other organisms to flourish.

Media for the Isolation of Pathogenic Members of the Coli-Typhoid Group

MacConkey's Bile-Salt Lactose Agar.—Commercial sodium taurocholate,[1] 0·25–0·5 gram (the exact quantity depends on the specimen of bile salts ; in some cases the larger amount may be inhibitory) ; sugar-free peptone, 2 grams ; tap water, 100 c.c. (if distilled water be used, 0·03 per cent. of calcium chloride should be added). The solution is steamed for two hours, filtered when hot, allowed to stand for twenty-four hours or till sedimentation has occurred, and filtered again. 1·5 to 2 per cent. agar is dissolved in the stock solution in the autoclave, if necessary cleared with white of egg and filtered ; 0·25–0·5 c.c. of a fresh 1 per cent. solution of neutral red and 1 per cent. lactose are added (for special purposes other sugars may be substituted for lactose). When this medium is used for examining urine or fæces, plates are inocu-

[1] The product supplied by May & Baker, Dagenham, London, is satisfactory.

lated (see p. 89). The colonies of any organism giving rise to acid will be of a rose-red colour.

Endo's Medium.—

Ordinary meat extract (or 0·5 per cent of Lab-Lemco in water) .	1000 c.c.
Peptone (sugar-free) . . .	10 grams.
Sodium chloride . . .	5 ,,
Agar-agar fibre cut into small pieces	20 ,,

Mix the ingredients and heat in the autoclave at 115° C. for half an hour; render alkaline by the addition of 10 c.c. of 10 per cent. sodium carbonate. Cool to 50° C. and add the white of an egg Steam in the Koch for one and a half to two hours; filter while hot through Chardin's paper. To each 100 c.c. of filtrate add 1 gram lactose, 0·05 c c. of filtered saturated alcoholic solution of basic fuchsin, and 2·5 c.c. of freshly prepared 10 per cent. sodium sulphite solution. Sterilise in the Koch for twenty minutes on three successive days. When the medium is hot it appears pink in colour, when cold it is almost colourless. (Should the medium be pink when cold the amount of sodium sulphite present is insufficient.) The medium must be kept in the dark. Colonies of lactose-fermenters are red.

These media in the hands of a worker accustomed to their use will yield good results. MacConkey's medium is that most used by British workers, and it has the merit of being easily prepared. As the result of a considerable experience we have found it most useful and reliable.

Brilliant Green Enrichment Method (Browning, Gilmour, and Mackie).—In this method advantage is taken of the fact that brilliant green has a greater inhibitory effect on *B. coli* generally than on *B. typhosus* and especially the paratyphoid group. The amount of the dye necessary to bring about the desired result is not a fixed quantity in each case, as it depends on the number of organisms in the fæces and also on the organic matter. A number of dilutions of the dye are therefore used. Tubes of peptone water neutral to litmus paper (peptone 2 per cent. and sodium chloride 0·5 per cent.) each containing 10 c.c. are prepared. The brilliant green sulphate (zinc free) is used as a 1 : 10,000 solution in distilled water. To three tubes of peptone water, 0·7, 0·4, and 0·25 c.c. of the brilliant green solution are added in series. Each tube is then inoculated with several large loopfuls of fæces (where the fæces are not fluid a thick suspension is made in sterile saline), and the tubes are incubated at 37° C. for twelve to twenty-four hours. At the end of this time a loopful is taken from each tube and strokes are made on plates of MacConkey's medium—three strokes with each loopful. Two plates will be sufficient for the strokes from all the dilutions. After incubation for another twenty-four hours the plates are examined for pale colonies. Often a pure culture of typhoid or paratyphoid bacilli is obtained from one of the dilutions. The method is not suitable for the isolation of Flexner or Sonne dysentery bacilli.

While *B. coli* generally is inhibited by the brilliant green, some

strains, especially the inosite-fermenters, e.g. *B. lactis aerogenes*, are equally resistant with *B. typhosus*; in cultures from the fæces of young children "paracolon" bacilli tend to be enriched by brilliant green. Many of these resistant organisms may be eliminated by adding 0·4 c.c. of a 1 : 1000 solution of telluric acid to the tubes of peptone water along with the varying amounts of brilliant green.

Müller's Tetrathionate Medium for the enrichment of organisms of the typhoid-paratyphoid group in fæces (Pesch and Kortenhaus):

(*a*) 900 c.c. sterile broth (pH 7·4);
(*b*) 50 grams chalk sterilised in the autoclave;
(*c*) 50 grams sodium thiosulphate crystals dissolved in water to make 100 c.c. and sterilised in the Koch;
(*d*) 5 grams iodine ground in a mortar with 4 grams potassium iodide and dissolved in sufficient water to make 20 c.c. —sterilisation is unnecessary.

The constituents are mixed and, without further heating, distributed in amounts of 10 c.c. in sterile plugged test tubes. A tube is inoculated with fæces and, after twenty-four hours' incubation, subcultures are made on a plate of MacConkey's or Endo's medium.

Wilson and Blair's Agar Medium [1] contains glucose, sodium sulphite, a soluble bismuth salt, sodium phosphate and ferrous sulphate with or without brilliant green. As the preparation of the medium is elaborate, reference should be made to the original description. It has given excellent results in the isolation of *B typhosus*, *B. paratyphosus*, *B.* and *B. enteritidis* (Gaertner) from fæces and sewage The rationale of its action is stated to be that in the presence of glucose *B. typhosus* reduces sulphite to sulphide, the colonies being blackened as seen after about thirty-six hours' incubation; also, bismuth sulphite which is formed, inhibits *B. coli* in the presence of suitable excess of sodium sulphite.

Plates should be poured and used within three or four days.

Media for the Isolation of Diphtheria Bacilli

Horgan and Marshall's Medium.—To 15 c.c. ordinary 2 per cent. agar medium with a pH of 7·6, melted and brought to 50° C., add 3·0 c.c. of a mixture of equal volumes of blood (from ox or horse, which has been defibrinated or rendered incoagulable by the addition of 1 per cent. sodium citrate) and of a 2 per cent. solution of potassium tellurite [2] in distilled water and pour into plates. The blood-tellurite mixture keeps in the ice-chest without aseptic precautions for three weeks and is most effective when at least three days old. Parish recommends the addition of 0·5 per cent. glucose to the digest broth basis used for the preparation of the agar.

McLeod's Medium.—This medium is used chiefly for the isolation and identification of *mitis* and *gravis* types of diphtheria colonies. 750–1000 grams of minced meat are extracted with 1000 c.c. tap-water kept at 48° C. for one hour. The fluid is then squeezed out through lint, left in the ice-chest overnight, and then filtered through paper. To 1000 c.c. filtrate add 20 grams peptone (Parke,

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[1] The medium is obtainable commercially.

[2] This is obtainable from British Drug Houses. A 1·5 per cent. solution is often preferable, being less inhibitory.

Davis & Co.) and 5 grams NaCl ; warm at 45° C. till solution occurs Test the reaction by taking 50 c c. and heating to 80°–90° C. for fifteen minutes, filtering through paper and finding how much N/10 NaOH is needed to bring 10 c.c. to pH 7·6. On this basis add sufficient alkali to bring the whole of the fluid to this reaction. Filter through a Seitz K "clarifying" disk ; then filter again through a Chamberland candle which has been sterilised in the autoclave. Distribute the filtrate in flasks or tubes, several of which should be kept for three days at 30° C. to control sterility. Store the rest in the ice-chest. To prepare the medium mix equal parts of this broth and 5 per cent. solution of agar in water which has been previously melted ; then add 7–10 per cent. of fresh, defibrinated rabbit blood and 0·04 per cent. of potassium tellurite. Heat the mixture at 75° C. for ten to fifteen minutes and pour into plates.

Smith's Method.—The following medium, containing telluric acid, is valuable for the isolation of diphtheria bacilli. It has the composition :

Peptone-water agar (neutral to litmus) . .	100 c.c.
Sheep's serum (sterilised at 57° C.) . . .	5 ,,
1 per cent. telluric acid solution in distilled water	0·9 ,,

The serum is added to the melted agar at a temperature of 50° C. On this medium the diphtheria bacillus usually forms large white colonies after incubation for twenty-four hours. The growth of many organisms is inhibited.

Media for Growing Fungi

1. *Beer Wort Agar* (Biourge).—Take beer wort as obtainable from the brewery (of a pale colour and without added hops) and dilute it till it has a s.g. of 1100. Add 1·5 per cent. of powdered agar, and heat in the Koch till it is dissolved (usually about two hours are necessary). Filter rapidly and fill into tubes. Sterilise in the Koch for twenty minutes on three successive days. If the medium is heated too long it loses the capacity of solidifying.

2. *Sabouraud's Medium* :

Pure tap water	1000 c c.
Maltose (" brute de Chanut ")[1] .	40 grams.
Peptone (" granulée de Chassaing ") .	10 ,,
Agar	18 ,,

In order to secure uniformity of results over a long series of observations, it is advisable to make up this medium in large quantities, say three litres at a time. According to Langeron the preparation is as follows. Mix the constituents in a large flask, then place in the cold autoclave and heat slowly until 120° C. is reached ; then cease heating. When the temperature has fallen to 100° C. filter quickly through a compact layer of moistened absorbent cotton-wool in several large filter funnels, all having been previously well heated with hot water. While the filtered medium is still hot it is distributed in tubes and flasks and sterilised

[1] Glucose (" massée de Chanut ") is sometimes substituted for maltose.

by placing in the cool autoclave and raising the temperature very slowly to 120° C., at which point heating is stopped. The medium must not solidify in the course of preparation. The study of the characters of the large colonies of trichophyta, etc., is best carried out with media distributed in 250 or even 500 c.c. Erlenmeyer flasks in which the requisite surface of medium with a suitably moist atmosphere is obtained.

3. *Carrot* serves as a good medium for the growth of moulds. Slices of carrot, shaped as in the case of potato (p. 71), are placed similarly in tubes and sterilised for twenty minutes at 120° C.

4. *Hanging-drop culture of moulds.*—An india-rubber washer $\frac{3}{20}$ inch high, about $\frac{7}{10}$ inch internal diameter, and of a size to lie completely on a slide, is luted on with lanolin, and a drop of water is placed on the slide within the ring. Then a large drop of culture fluid, *e.g.* 1 per cent. glucose broth, is placed on a thin sterile slide and inoculated with the mould. The slide with the drop is inverted over the rubber ring, the surface of which has been smeared with lanolin, so that an air-tight cell results.

5. *Isolation of Aspergillus from sputum.*—Inoculate a portion of the sputum into a flask containing 2 per cent. glucose and 1 per cent. glycerol in water. Incubate for forty-eight hours at 37° C.

The Observation of Bacterial Fermentation of Sugars, etc.—The capacity of certain species of bacteria to ferment sugars constitutes an important biological property, which is utilised for their classification and differentiation. As a result of this action the fermentable substance is broken down and acid products are formed, and also frequently gases. Besides sugars, closely allied bodies which are alcohols, and similarly glucosides (which are combinations of glucose with other substances) and other compounds (*e.g.* inosite) may also be acted on by bacteria. It is essential to make sure that pure cultures are used for fermentation tests, since a mixture of several species may exhibit "synergic action," gas being then produced from a substance which does not yield gas when acted on by each organism alone. The following fermentable substances are commonly employed:

Monosaccharides.—*Pentoses*—arabinose (obtained from gum arabic), xylose (from wood), and rhamnose (which is really a methylpentose). *Hexoses*—glucose (dextrose), lævulose, mannose (from the vegetable ivory nut), and galactose (a hydrolytic derivative of lactose).

Disaccharides.—Lactose, saccharose, maltose.

Trisaccharides.—Raffinose.

Polysaccharides.—Starch, inulin (from dahlia roots), dextrin, arabin, glycogen, cellulose.

Alcohols.—Glycerol, erythritol, adonitol, mannitol, dulcitol, sorbitol.

Glucosides.—Salicin, coniferin, aesculin.

The end products of bacterial fermentations may be various. The tests usually employed for the detection of ordinary fermentative processes depend on two kinds of changes, namely: (*a*) the formation of acids, and (*b*) the evolution of gases (chiefly carbon dioxide, hydrogen, and methane). Generally speaking, these tests are reliable, and the methods to be pursued are simple. For the estimation of other products, alcohols, etc., and the identification of the acids complicated chemical procedures may be necessary. Besides such gases as those named some organisms give rise to sulphuretted hydrogen by breaking down the protein.

In testing the effect of a bacterium on a given sugar it is essential that this sugar alone be present; the basis of the medium ought therefore to be either peptone solution or casein digest broth (p. 58). In the case of organisms requiring serum for their growth Hiss's serum water medium is used, but other workers prefer peptone water agar to which 5 per cent. of sterile serum has been added. The sugar or other substance is added in the proportion of from a half to 1 per cent. If the sugar in solid form be placed in the broth and this then sterilised, there is danger that chemical changes may take place in the sugar, in consequence of its being heated in the presence of substances (such as alkalis) which may act deleteriously upon it. Therefore it is preferable that the addition should be made in the form of a sterile 10 per cent. solution in water—sterilisation being effected by autoclaving for ten minutes at 115° C. or by passing through a Seitz filter (p. 99). In any case, the completed medium should not be heated above 100° C.

The *development of an acid reaction* in the medium is demonstrated by the previous addition of an indicator. In Hiss's serum water media the production of acid also leads to coagulation of the medium. Sometimes acid is formed very slowly from sugars, so that it is well to keep the cultures under observation for several days or even longer.

Acid and gas formation may be simultaneously tested for, by placing the fluid medium containing the indicator in Durham's tubes.

In all tests in which sugars are used, a control uninoculated tube ought to be incubated along with the bacterial cultures, as changes in reaction sometimes occur spontaneously in media containing unstable sugars. Tests in which sugars are used are best carried out in alkali-free glass tubes.

Hiss's Serum Water Media.—These are composed of one part of serum (*e.g.* ox's) and three parts of distilled water with 1 per cent. litmus or Andrade's indicator; various sugars in a pure condition are added in the proportion of 1 per cent. The development of acid by fermentation is shown by the alteration of the colour and by coagulation of the medium. These media do not coagulate at 100° C., and thus can be sterilised in the steam steriliser, the same precautions being observed as in the case of other media containing sugars. They have been extensively used in studying the fermentative properties of streptococci and pneumococci, etc. (As controls, cultures of the organisms should be made in the same specimen of serum water medium from which, however, the sugar has been omitted.)

Indicators

Litmus.—To any of the ordinary media litmus (French, tournesol) may be added to show change in reaction during bacterial growth The litmus is added, before sterilisation, as a strong watery solution (*e.g.* the Kubel-Tiemann solution), in sufficient quantity to give the medium a distinctly bluish tint.[1] During the development of an acid reaction the colour changes to pink, and may subsequently be discharged. The disadvantage of litmus is that the colour change with it is not very sharp.

Neutral-Red.—This dye was introduced by Grünbaum and Hume as an aid in determining the presence or absence of members of the *B. coli* group, especially in the examination of water. The media found most suitable are agar or broth containing 0·5 per cent. of the sugar to be tested, to which 0·5 per cent. of a 1 per cent. watery solution of neutral-red is added. The alkaline medium is of a yellowish brown colour which in the presence of acid passes into a deep rose-red. Sometimes there subsequently occurs a change to a fluorescent green, caused apparently by a change in the composition of the dye, as the fluorescence is not discharged by addition of alkali. (See also p. 511.)

Andrade's Indicator.—An aqueous 0·5 per cent. solution of acid fuchsin is decolorised by the addition of N/1 NaOH. About 16 c.c. are required for 100 c.c. of the dye solution: if the mixture is still red after standing for three hours 1 c.c. further of the caustic soda solution should be added; 1 per cent. of this mixture is added to media. Media with a pH 7·2 are red when hot, but faintly yellow when cold. Acid-formation restores the red colour.

Bromcresol Purple.—The sulphone-phthalein compound bromcresol purple is a useful indicator. It has a purple colour in neutral or alkaline solution, which becomes yellow when the reaction is very slightly acid; hence it is a delicate indicator for detecting acid production: 1 c.c. of a 1·6 per cent. alcoholic solution is added to 1000 c.c. of medium.

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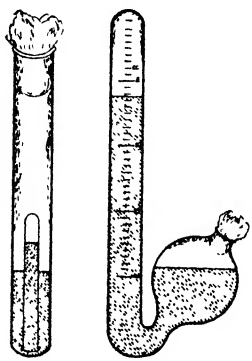
[1] The litmus solution is made as follows: Solid commercial litmus is digested with pure spirit at 30° C. till on adding fresh alcohol the latter becomes only of a light violet colour. A saturated solution of the residue is then made in distilled water and allowed to stand until the sediment settles or it may be centrifuged. When this is diluted with a little distilled water it is of a violet colour, which further dilution turns to a pure blue. To such a blue solution very weak sulphuric acid (made by adding two drops of dilute sulphuric acid to 200 c.c. water) is added till the blue colour is turned to a wine-red. Then the saturated solution of the dye is added till the blue colour returns.

For *the observation of gas-formation* the following methods may be employed :

(1) *Durham's Tube* (Fig. 9, *a*).—A small test-tube is inverted and slipped down into the empty culture tube, which is then plugged and sterilised in the autoclave. The medium, which has been previously sterilised, is then tubed with aseptic precautions and the tubes are finally heated in the Koch for fifteen minutes on each of three successive days. The air remaining in the smaller tube is thereby expelled. The tube is then inoculated with the bacterium to be tested. Any gas developed collects in the upper part of the inner tube. As some of the sugars now used for fermentation tests are expensive, it is well to arrange the Durham apparatus with very small tubes ; with these a satisfactory result can be obtained with only 1 c.c. of medium.



a *b*

FIG. 9.—Tubes demonstrating gas-formation by bacteria.

a, Durham's fermentation tube.
b, Smith's fermentation tube.

(2) *Smith's Fermentation Tube* (Fig. 9, *b*).—This consists of a tube of the form shown, and the figure also indicates the extent to which it ought to be filled. It is inoculated in the bend with the gas-forming organism, and when growth occurs the gas collects in the upper part of the closed limb, the medium being displaced into the bulb. If the limb be graduated the amount of gas evolved can be measured, and rough chemical tests can be applied, *e.g.* the presence of carbonic acid gas can be tested for by absorbing it with a solution of caustic soda, and that of hydrogen by ignition (see under *B. coli*).

In certain cases it is desirable to ascertain the final pH reached when an organism has grown in fluid medium containing a sugar. For this purpose the initial pH is adjusted and the medium inoculated ; then after the culture has been kept for a standard time the pH can be ascertained with sufficient accuracy for most purposes by testing portions with a universal indicator mixture or with a series of indicators which have overlapping ranges.

The titratable acidity may also be estimated in the usual way by taking a measured volume of culture and, with phenol phthalein as indicator, finding how much N/10 NaOH is required to restore the reaction to that of the original medium.

The Observation of Indole-formation by Bacteria.—The formation of indole from protein by a bacterium sometimes constitutes an important specific characteristic. To observe indole production the bacterium is grown, preferably at 37° C., in a fluid medium containing peptone. The latter may either be peptone water (p. 58) or casein digest broth (p. 58)—the

tryptophane being the component from which indole is formed. Any medium containing sugars must be avoided, as their presence may inhibit the production of indole.

The amount of indole produced by a bacterium seems to vary very much with certain unknown qualities of the peptone. It is well, therefore, to use a specimen which yields a good reaction with a culture of a known indole-producer, *e.g.* *B. coli*. In general, a well-grown forty-eight hours' culture is used for the test, but an older culture may be necessary, *e.g.* seven days.

(1) *Ehrlich's Rosindole Reaction.*—Böhme showed that for ease of application and delicacy of effect the reaction possesses great advantages. It depends on the fact that paradimethylamidobenzaldehyde unites with indole to form a rosindole body whose colour is readily developed, especially in presence of an oxidising substance such as potassium persulphate ($K_2S_2O_8$). Two solutions are required:

(1)	Paradimethylamidobenzaldehyde . .	4 grams.
	Absolute alcohol (96 per cent.) . .	380 c.c.
	Concentrated hydrochloric acid . .	80 ,,
(2)	Potassium persulphate . .	Saturated watery solution

To a culture of the organism in 5 c.c. of suitable fluid medium add 1 c c. of (1) and then 1 c c. of (2), and shake well; if indole be present a rose-red colour will appear in a few minutes. Sometimes the rose colour appears on the addition of solution (1), and the addition of a special oxidising agent is unnecessary. The rosindole compound can be separated from the culture by shaking the latter up with amyl alcohol, and this should be done in cases of a doubtful reaction, as sometimes when a faint pink colour appears in the culture tube the extracting alcohol remains colourless, showing that no real reaction has occurred; chloroform may also be used. Since various tryptophane derivatives may react positively, but indole is the only one which is volatile, Goré increased the specificity of the test by the following procedure: Moisten the under-surface of the plug of the culture tube, which must consist of white absorbent cotton-wool, with 4–6 drops of the persulphate solution and then with a few drops of the aldehyde solution. Then push the plug down the tube till it is 1–1½ inches above the surface of the culture. Place the tube upright in a boiling water-bath for fifteen minutes, avoiding any wetting of the plug by the culture medium. Remove the test tube and examine the under-surface of the plug, which in the presence of indole will be rose-coloured.

For the quantitative estimation of indole, Happold and Hoyle proceed as follows: 10 c.c. of culture acidified with one drop of concentrated HCl are extracted with two successive 10 c.c. amounts of light petroleum (B.P. 40°–60°). A third extraction may be made to ensure complete removal of indole. The pooled extracts are washed once with 10 c.c. of distilled water. The light petroleum is then extracted with successive 5 c.c. amounts of freshly prepared Ehrlich's reagent (1) until no more rosindole is produced. As the rosindole is completely insoluble in light petroleum it collects in the layer of Ehrlich's reagent. When the amount of indole is very small the reagent should be added 1 c.c. at a time. A standard

1/50,000 solution of indole is prepared in the same medium as the culture and treated similarly. The rosindole from the culture is then compared with that from the standard in Nessler tubes. Milkiness in the solutions is removed by immersing both tubes in boiling water for the same time, a few minutes being sufficient.

(2) *Gnezda's Test.*—A strip of filter-paper which has been dipped in warm saturated watery oxalic acid solution and allowed to dry is inserted into the culture tube so that it presses against the side and remains near the mouth. The culture is then incubated. The development of a pink tint in the oxalic acid crystals on the paper indicates the formation of indole. This test is stated to be highly specific for indole.

The Observation of Sulphuretted Hydrogen Production by Bacteria..—Strips of filter paper about $0{\cdot}5 \times 5$ cm. soaked in saturated lead acetate solution are autoclaved in a plugged tube and dried at 120° C. A broth or agar culture is inoculated with the organism to be tested and a strip of the paper is inserted into the mouth of the test tube so that nearly one-half projects beneath the cotton-wool plug. The culture is incubated and examined daily for darkening of the paper which indicates H_2S. It is important to use in the medium peptone which yields a positive result with a known producer of H_2S such as *B. coli.*

The Use of the Ordinary Culture Media and Methods of Making Cultures

The culture of bacteria is usually carried on in test-tubes conveniently $6 \times \frac{5}{8}$ inches, but for many purposes smaller tubes, $5 \times \frac{1}{2}$ inches, are equally suitable and medium is thus saved. The tubes ought to be very thoroughly washed and dripped, and their mouths plugged with absorbent cotton wool.[1] They are then sterilised for one hour at 170° C. If the tubes be new, the glass, being usually packed in straw, may be contaminated with the extremely resistant spores of the *B. subtilis*. Cotton-wool plugs are universally used for protecting the sterile contents of flasks and tubes from contamination with the bacteria of the air. A medium thus protected will remain sterile for years. Whenever a protecting plug is removed for even a short time, the sterility of the contents may be endangered. It is well to place the broth, gelatin, and agar media in the test-tubes directly after filtration. The media can then be sterilised in the test-tubes.

In filling tubes it is convenient to employ as a reservoir for the medium, a large glass funnel to which a glass nozzle is attached by rubber tubing (all the parts should be sterilised before use); to prevent contamination from the air the sterile lid of a Petri plate should be laid over the mouth of the funnel. In filling

[1] *See* footnote, p. 50.

tubes, care must be taken to run the liquid down the centre, so that none of it drops on the inside of the upper part of the tube with which the cotton-wool plug will be in contact, otherwise the latter will subsequently stick to the glass and its removal will be difficult. In the case of liquid media, test-tubes are filled about one-third full. With the solid media the amount varies. In the case of gelatin media, tubes filled one-third full and allowed to solidify while standing upright, are those commonly used. With organisms needing an abundant supply of oxygen the best growth takes place on the surface of the medium, and for practical purposes the surface ought thus to be as large as possible. To this end "sloped" agar and gelatin tubes are used (Fig. 10, *b*). To prepare these, tubes are filled only about one-sixth full, and after sterilisation are allowed to solidify lying on their sides with their necks supported so that the contents extend 3 to 4 inches up, giving an oblique surface after solidification. Thus agar is commonly used in such tubes (less frequently gelatin is also "sloped"), and this is the position in which blood serum is inspissated. Tubes, especially those of the less commonly used media, should be placed in large jars provided with stoppers, or the medium be kept in bottles provided with screw-caps, otherwise the contents are apt to evaporate. Medium which has been inoculated with a bacterium, and on or in which growth has taken place, is called a "culture." A "pure culture" is one in which only one species is present. The methods of obtaining pure cultures will presently be described. When a fresh tube of medium is inoculated from an already existing culture, the resulting growth is said to be a "subculture" of the first. Manipulations involving the transference of small portions of growth either from one medium to another, as in the inoculation of tubes, or, as will be seen later, to cover-glasses for microscopic examination, are effected by

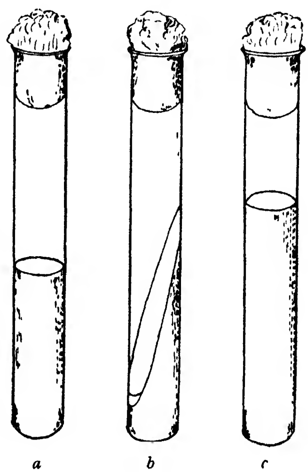


FIG. 10.—Tubes of media.

a. Ordinary upright tube
b. Sloped tube.
c. "Deep" tube for cultures of anaerobes.

pieces of platinum wire (Nos. 26 or 28 Imperial Standard wire gauge—corresponding to 0·457 and 0·381 mm. thickness) fixed in glass rods 8 inches long, or in aluminium holders. If platinum wire is not available an excellent substitute is found in "resistance wire," No. 27 I.S.W.G. This is best mounted in an aluminium handle. Every worker should have two wires. One is 2½ inches long, and has a loop turned upon it (Fig. 11*a*); this is referred to as the platinum "loop" and is used for many purposes—"taking a loopful" is a phrase constantly used. The other wire (Fig. 11*b*) ought to be 4½ inches long and straight; it is used for making stab cultures. It is also very useful to have at hand a platinum-iridium spud. This consists of a piece of platinum-iridium about 1½ inches long, 1·3 mm. broad, and of sufficient thickness to give it a firm consistence; its distal end is expanded into a diamond shape and its proximal is screwed into an aluminium rod. It is very useful for making scrapings from organs and for disintegrating felted bacterial cultures; in such manipulations the ordinary wire bends too easily.

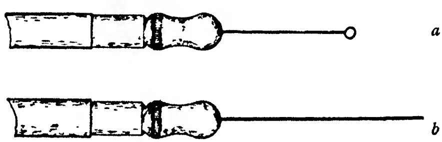


FIG. 11.—Platinum wires in aluminium handles. (Reduced in size.)

a Platinum "loop."

b. Straight wire for ordinary puncture inoculations.

If a platinum wire heavily charged with bacteria be sterilised in a Bunsen flame "sparking" may occur and unkilled bacteria thus fall on the worker's bench. In working with organisms highly pathogenic to man, *e.g.* those of glanders, plague, Malta fever, it is well to substitute for platinum needles glass rods drawn out to capillary diameter, each of which can be destroyed after use. These before use are sterilised by passing through the flame, and when contaminated are dropped into a 1–1000 solution of corrosive sublimate instead of being heated.

Cultures on a solid medium are referred to (1) as "puncture" or "stab" cultures, or (2) as "stroke" or "slant" cultures, according as they are made on medium (1) solidified in the upright position, or (2) sloped. Stab cultures, *e.g.* in agar, are suitable for maintaining organisms alive for long periods, since they dry up less quickly than stroke cultures.

To make a Subculture on an Agar "Slope" from another Culture.—The two tubes are held between the thumb and first two fingers of the left hand, in a slanting position, toward their lower ends, with the sloped surfaces upward. Then with the right hand rotate

the cotton-wool stoppers of both tubes, so that they may be readily removable. Take the end of the holder of the looped wire between the thumb and first two fingers of the right hand. Sterilise the wire by holding it vertically in the Bunsen flame. Remove the stopper of the tube from which the inoculation is to be made with the crooked little finger of the right hand and retain it there; flame the mouth of the tube. Pass the needle into the tube and touch the medium to ensure that the wire is cool (if the wire is too hot it will be seen to melt the medium; one must then wait for a few seconds before proceeding). Touch the growth with the wire, which is then withdrawn; remove the stopper from the second tube with the points of the thumb and index finger and retain it there; insert the wire charged with growth and smear lightly the surface of the agar (avoid cutting into the medium). Withdraw the wire and sterilise it in the flame as before; flame the mouths of the tubes and replace the stoppers in their respective tubes.

To make a Stab Culture.—Use the straight wire charged with

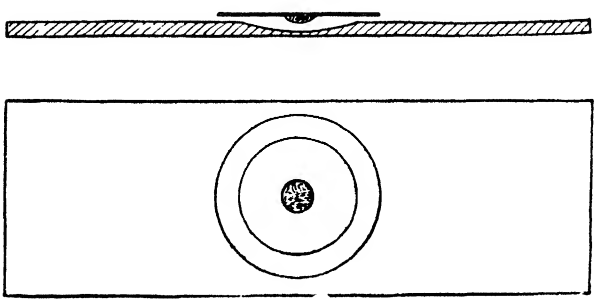


FIG. 12.

Hollow-ground slide for hanging-drop cultures, shown in section and plan

culture and pass it into the centre of the medium, taking care to withdraw it in the same track so as to avoid splitting the medium.

To inoculate fluid medium a loopful of culture is deposited on the wall of the tube slightly above the level of the fluid and then the tube is tilted so as to wash the material down. If the inoculum is tenacious, it is rubbed on the wall of the tube, several loopfuls of the contents being incorporated with it by means of the wire, so that a uniform suspension is produced. When large quantities of culture are required, it is often advisable to use bigger inocula. This is the case also when delicate organisms are being subcultured.

Hanging-drop Preparations.—It is often necessary to observe micro-organisms alive, either to watch the method and rate of their multiplication, or to investigate whether or not they are motile. For the latter purpose a "wet preparation" is often sufficient; this consists of a drop of fluid containing the organisms, which is allowed to spread into a thin film between a slide and cover-glass. For more extended study it is necessary to make hanging-drop cultures. The method in the form to be described is only suitable for aerobes. For this a special slide is used (Fig. 12). There is

ground out on one surface a hollow having a diameter of about half an inch. The slide and a cover-glass are sterilised by hot air in a Petri's dish, or simply by being heated in a Bunsen and laid in a sterile Petri to cool. One or other of two manipulation methods may be employed. (1) If the organism be growing in a liquid culture, a loop of the liquid is placed on the middle of the under-surface of the sterile cover-glass, which is held in forceps, the points of which have been sterilised in a Bunsen flame. If the organism be growing in a solid medium, a loopful of sterile broth is placed on the cover-glass in the same position, and a *very* small quantity of the culture (picked up with a platinum wire) is rubbed up in the broth. The cover is then carefully lowered over the cell on the slide, the drop not being allowed to touch the wall or the edge of the cell. The edge of the cover-glass is covered with vaseline, and the preparation is then complete and may be placed under the microscope. If necessary, it may be first incubated and then examined on a warm stage. (2) The sterile cover-glass is placed on a sterile glass plate. The drop is then placed on its *upper* surface, the details being the same as in the last case. The edge of the cell in the slide is then painted with vaseline, and the slide, held with the hollow surface downwards, is lowered on to the cover-glass, to the rim of which it of course adheres. The slide with the cover attached is then quickly turned right side up, and the preparation is complete.

It is sometimes convenient for the observation of the growth of bacterial colonies or of fungi to make hanging-drop cultures with a solid medium. This can be done by substituting a drop of melted gelatin or agar for broth and inoculating the surface after solidification, *see also* p. 77. The method of microscopic examination is described on page 107.

Cultures of Bacteria from Single Cells.—A number of methods have been devised for the purpose of obtaining pure cultures starting from a single organism. Burri's india-ink method consists in emulsifying the organisms in dilute india-ink and depositing by means of a fine pen a number of minute droplets of the mixture on the surface of nutrient medium in a Petri plate. The drops are covered with a cover-slip and are examined under the microscope and those which contain a single bacterium are noted. Time is allowed for multiplication, and a subculture is then made from colonies which have developed from single organisms. With a view to rapid working and to obviating the use of the ink, which is harmful to some bacteria, Ørskov has modified the method as follows: A layer of agar several mm. thick is poured into a flat Petri plate and a suitable loopful of a twelve-hour broth culture, *e.g.* of *B. coli*, is spread over the medium by means of a bent glass rod. The plate is then placed in the incubator for one hour, at the end of which time multiplication is just beginning in the case of *B. coli* and the organisms are readily distinguishable under the microscope. A block of agar is excised and its under side placed on a microscope slide which has previously been sterilised by flaming. The inoculated surface of the agar is examined under the microscope and a field is selected which contains only a single organism. In order to enable the same area to be found again some form of object finder is required for rough orientation; in addition to this, closely spaced crossing lines have previously been cut on the under side of the slide by

scratching with a diamond through a drop of immersion oil ; by means of these along with an eyepiece micrometer it is possible to register the field exactly, and the position is recorded by careful drawings of the lines. The slide is now placed in a Petri capsule in the incubator, the atmosphere being kept moist by inserting a piece of wet filter-paper in the capsule ; great care, however, must be taken that the preparation does not become too moist or the agar block will become displaced on the slide. When a colony has developed from the single organism a subculture is made. To do this a piece of fine platinum wire is attached to the front lens of an objective by means of modelling wax, and its point is lowered on to the colony and the organisms which adhere are used to inoculate a tube of broth. (It is necessary to ascertain first of all the exact point in the field which the needle-point impinges on. To do this a small drop of india-ink is placed on an agar block similar to that bearing the culture and the needle is lowered on to the agar ; the point in the field occupied by the puncture mark is then noted with the microscope and the colony is shifted into this position.) The method has been modified by Gardner.

The method of Topley, Barnard and Wilson is as follows. A loopful of a young broth culture of the organism (*e.g.* after six hours' incubation) is used to inoculate a tube of 10 per cent. gelatin in 1 per cent. peptone water previously melted and cooled to 37° C. This culture is kept for two hours in the incubator ; then a loopful (external diameter of loop 3 mm.) is placed on a sterile circular quartz coverslip of 12 mm. diameter which is inverted on a sterile slide so as to yield a thin film free from bubbles and not exuding at the edge. By examination with dark-ground illumination under a dry $\frac{1}{6}$ inch objective, a well-isolated organism toward the centre of the coverslip is selected. Under the low power a droplet of mercury is picked up with a rough, rusty steel needle, placed on the coverslip, and then pushed along with a needle until the selected organism is covered. The preparation is then exposed to a suitable source of ultra-violet light for a time sufficient to kill all the other bacteria, that under the mercury being protected. By way of control similar preparations without drops of mercury are exposed to the light in the same way. These should prove sterile on subsequent incubation at 25° C., whereas the screened organism, if viable, will have formed a small colony. A subculture is then made from the latter.

Another method consists in the use of a micro-manipulator such as that of Schouten.[1] A difficulty in such work is that isolated organisms may fail to yield growths. (For details, reference must be made to the original papers.)

INOCULATING ROOM.—Where there is special liability to contamination from atmospheric dust some form of inoculating room or hood should be used in which to make cultures. In its simplest form this consists of a large oblong box which sits on end on the bench. It has windows on three sides and the fourth side, corresponding with the edge of the bench, consists of curtains of waterproof material. Ventilation spaces are covered with fine

[1] Supplied by Dr. S. L. Schouten, Hygienic Laboratory, The University, Utrecht.

metal gauze ; and electric light and a Bunsen burner are provided. The inner surface is freed from dust by wiping with a cloth soaked in antiseptic solution and about half to one hour before use the interior is well sprayed with water from a fine spray so as to remove floating dust as far as possible.

The Separation of Aerobic Organisms in Cultures—Plate Cultures

The general principle underlying the methods of separation is the distribution of the bacteria in or on one of the solid media so that the colonies formed by the individual organisms are sufficiently far apart to allow their being examined separately. For the purpose, circular shallow glass capsules, each fitted with an overlapping glass cover, are almost universally used ; these are known as Petri's dishes or capsules (Fig. 13). The medium, after being melted, is poured into a sterile capsule and allowed to solidify, so as to form a thin layer ; in this way the colonies which afterwards grow are readily accessible. In one method the material containing the bacteria is smeared over the surface of the medium after it has solidified in the capsule — "method of successive strokes." In another method the organisms are mixed with the medium when in the melted state, and the mixture is then poured into the capsule and allowed to solidify—"dilution method."

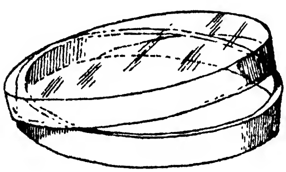


Fig. 13.—Petri's capsule. (Cover shown partially raised.)

The method of successive strokes is the more convenient, and is that used for the separation of typhoid and dysentery bacilli, meningococcus, etc. ; it is, in fact, capable of almost universal application. The procedure varies according to the material to be examined, but in all cases it is essential to have the surface of the medium dry. This is secured by removing the lid of the capsule when the medium has solidified and covering the plate with sterile paper, after which it is placed in the incubator for half an hour, or drying may be effected more rapidly by warming the open plate quickly over a Bunsen flame (taking care, however, not to melt the medium) and then covering with paper until cooling has occurred. If the organisms are on a swab, say from the naso-pharynx, consecutive strokes are made all over the surface of the medium, always the same portion of the swab being brought into contact with it. In this way the organisms are gradually wiped off the swab, till in the later

strokes they may be deposited at sufficiently wide intervals to give separate colonies. Agar plates should always be incubated in the inverted position to prevent contamination of the surface by condensation water from the lid of the plate. Plates of gelatin, however, are kept with the medium side down, as liquefaction often occurs from the growth of organisms. Sometimes it is advisable to smear several plates consecutively with the same portion of the swab. If the material to be examined is fluid, *e.g.* an emulsion of fæces, a usual method is to place a loopful on the surface of the medium, and then, with a sterile glass rod bent at a right angle, to smear the whole surface. If the organisms are found, on microscopic examination, to be very numerous, say in pus, it will be advisable to dilute with sterile saline before making the smears. The characters of the colonies which appear on the plates can be examined with a hand-lens, magnifying about 6 diameters. In some cases examination under a low-power of the microscope is an advantage ; the plate in the inverted position can be put on the stage of the microscope for this purpose (p. 91). For the culture of special organisms, as afterwards detailed, the agar or other medium is smeared with sterile serum or blood according to the growth requirements of the organism, or the serum or blood is added before the medium is poured.

When making cultures from emulsions of fæces in order to recover organisms of the enteric and dysentery groups, it is important to obtain the largest possible number of isolated colonies. The following method has been found very effective (Fig. 14). A loopful of fluid fæces or of fæcal emulsion is spread on the medium at one side of the plate in the area A ; then, without recharging the needle, successive series of parallel strokes are made in different directions (*e.g.* a series in the direction indicated by the lines B, a second series in the direction shown by the lines C, and so on)—the flat part of the loop being throughout kept in contact with the medium. Plates with a diameter of 4 or 6 inches are useful ; as the layer of medium must not be too thin about 15 to 40 c.c. is sufficient respectively.

The principle just described may be applied also to agar in tubes, but the results generally are not so satisfactory, and the characters of the colonies cannot be so readily studied. In this case several agar tubes are taken, a platinum loop is charged with the material to be examined, and in each tube several vertical strokes are made from below upwards on the surface of the agar, one tube after the other being used without recharging the needle. The tubes after inoculation should be kept in the upright position, so that the water of condensation is not allowed to run over the surface.

Dilution Method.—In this method the bacteria are added to

the medium when liquid, and mixed by rolling the tube between the palms of the hands (to avoid shaking, which causes air bubbles); the inoculated medium is then poured out into a capsule (*i.e.* "plated") and allowed to solidify. As in this case the organisms are distributed throughout the medium, some of the colonies grow on the surface of the medium—"superficial colonies"—others in its substance—"deep colonies." These often show different appearances, which are sometimes used in the systematic description of an organism. As the bacteria may

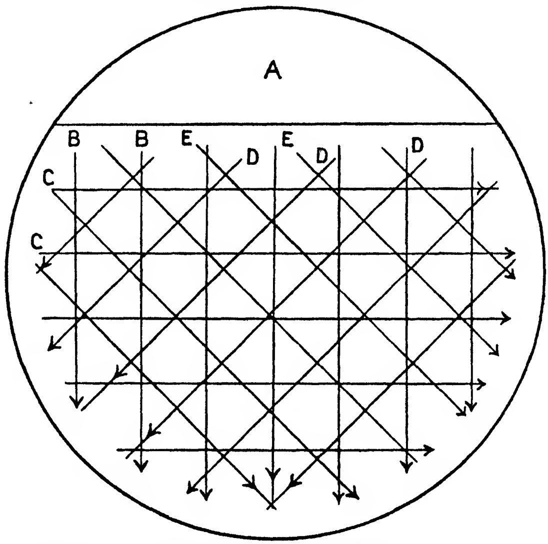


FIG. 14.—Diagram of method of inoculating plate from fæces. (After Medical Research Council's Special Report No. 51.)

produce too many colonies to allow separation, means must also be used for making different dilutions, a separate plate being prepared for each. If gelatin is used, the medium in tubes is melted and kept in a beaker of water at about 28° C. If agar is used, the medium is melted thoroughly by boiling in a vessel of water and then allowed to cool to about 43° C., at which temperature the inoculations are made. The following are the details:

The contents of three tubes, marked *a*, *b*, *c*,[1] are liquefied as above described. Inoculate *a* with the bacterial mixture. The amount of the latter to be taken varies, and can only be regulated by ex-

[1] For marking glass vessels it is convenient to use the red, blue, or yellow grease pencils specially made for the purpose.

perience. If the microscope shows enormous numbers of different kinds of bacteria present, just as much as adheres to the point of a straight platinum wire is sufficient. If the number of bacilli is small, one to three loops of the mixture may be transferred to the medium. Mix the contents of *a* well. Transfer two loops of medium from *a* to *b*. Mix *b* and transfer five loops to *c* and mix. The plugs of the tubes are in each case replaced and the tubes returned to the beaker. The contents of the three tubes are then poured out into three capsules. In doing so the plug of each tube is removed and the mouth of the tube passed two or three times through the Bunsen flame, the tube being meantime rotated round a longitudinal axis. Any organisms on its rim are thus killed. The capsules are labelled and set aside till growth takes place.

For accurate work it will be found convenient to carry out the dilutions in definite proportions as follows: In a number of small sterile test-tubes 0·95 c.c. sterile water or broth is put. To the first tube we add 0·05 c.c. of the bacterial mixture. The contents of the tube are well shaken up, and the pipette is sterilised by boiling in water. It is allowed to cool, and 0·05 c.c. of fluid is transferred from the first tube to the second. By a similar procedure 0·05 c.c. is transferred from the second to the third, and so on. There is thus effected a twenty-fold dilution in each successive tube. After these steps have been carried out, a definite amount, say 0·05 c.c., is transferred from each tube to a tube of melted medium,—the medium being afterwards plated and the colonies counted when growth occurs. The number of tubes required will vary according to the number of bacteria in the original mixture, but usually four or five will be sufficient.

Enumeration of Colonies.—The dilution method just described supplies the means of counting the number of living bacteria in a fluid, the proviso being always made that they are capable of growth in the medium used. For pathogenic organisms one of the agar media is generally used; in the case of water, gelatin may also be employed. The dilutions are made by the quantitative method, and a given amount, say 0·1 c.c., is taken from one of the dilutions and transferred to a tube of melted medium, and, after gentle mixing, the medium is poured in a Petri capsule. It is advisable to take samples in this way from two or even three of the dilutions. To aid the counting of the colonies which develop, various patterns of ruled glass plates have been introduced. If the ruling is in the form of squares of given size, the number of colonies in several squares is counted, and as the area of the Petri dish can be got by multiplying the square of its radius by $3\frac{1}{7}$, the whole number can then be calculated. Petri dishes are rarely flat, and unequal distribution of the colonies has accordingly to be taken into account. The dilution to be selected for taking the sample for plating will depend upon the relative abundance of the organisms in the original fluid.

Plate Culture Microscope.—In the examination of plate

cultures a low-power binocular microscope is useful. By means of it one may study the characters of individual colonies, especially when they are small; also the making of subcultures from these is facilitated. A magnification of ten is generally useful.

Separation by Killing Non-spored Forms by Heat.—As has been said, the spores of bacteria resist heat more than the vegetative forms. When a mixture contains spores of one bacterium and vegetative forms of this and other bacteria, then if the mixture be heated for thirty minutes at 70–80° C. all the vegetative forms may be killed, while the spores will remain alive and will develop subsequently. Several tubes of different media should be inoculated and treated thus, as the success of the method is very variable.

Separation of Pathogenic Bacteria by Inoculation of Animals.—It is found difficult, and often impossible, to separate by ordinary plate methods certain pathogenic organisms, such as *B. tuberculosis*, *B. mallei*, and the pneumococcus, when such occur in conjunction with other bacteria. These grow best on special media, and the first two grow so slowly that the other organisms present may outgrow them, cover the whole plates, and make separation difficult. The method adopted in such cases is to inoculate an animal with the mixture of bacteria, wait until the particular disease develops and death occurs, or kill the animal, and with all aseptic precautions (*vide* p. 164) inoculate tubes of suitable media from characteristic lesions situated away from the seat of inoculation.

The Principles of the Culture of Anaerobic Organisms

All ordinary media, after preparation, will contain traces of free oxygen, and will absorb more from the air on standing. Therefore media intended for the growth of anaerobes should always be heated at 100° C. for a few minutes, immediately before inoculation in order to remove the oxygen. It is advantageous if they contain a reducing agent which does not interfere with bacterial growth. Such an agent takes up any oxygen which may already be in the medium, and prevents further accumulation. The reducing body used is generally glucose, sodium formate (1 per cent.) or cysteine (0·02 per cent.). Reabsorption of oxygen can be minimised in the case of shake cultures in solid medium by using deep tubes. In the case of broth, *Noguchi's tubes* are employed.

These are long narrow tubes (8 × ½ inches) which are half filled with medium and are heated in the Koch for thirty minutes or in boiling water for five minutes. Melted vaseline (previously sterilised at 170° C.) is then poured on the surface of the medium and the tubes are cooled quickly. Inoculation is effected by means of a capillary pipette after melting the vaseline, which acts as a seal excluding the air. This method is not suitable for the culture of organisms which produce much gas, as the vaseline plug is forced out of the tube.

Another method is that of Harris. A small hole with smooth margins is blown at the tip of the closed end of a Smith's fermentation tube. It is then covered by a well-fitting viskap [1] which is allowed to dry slowly. The tube is now filled and sterilised as usual. After growth has occurred following inoculation the cotton-wool plug of the tube is replaced by an india-rubber stopper. Then by puncturing through the hole with a sterile needle and syringe a sample of the contents of the closed limb is obtained.

When surface growths on solid media are required, oxygen can be removed by (1) displacing it by an inert gas, usually hydrogen, (2) absorbing it with sodium pyrogallate, (3) combustion, or (4) bacterial growth. Combinations of several of these methods are frequently used. Anaerobes will grow without excluding the air if the medium contains constituents which lead to the disappearance of oxygen and exert a reducing action; this is effected by having portions of animal or vegetable tissue present. Sometimes fresh tissues which contain catalase have been employed, *e.g.* portions of kidney added to fluid medium; but in many cases heated tissues act well, as in Robertson's cooked meat medium, the mode of action of which has been demonstrated by Martin and Lepper. The disappearance of oxygen is chiefly due to auto-oxidation of unsaturated fatty acids of the lipins contained in the meat, the reaction being catalysed by the muscle hæmatin. Heart muscle is preferable because it contains about twice as much lipins as other flesh. In addition, the small amount of glutathione which is present and the fixed sulphhydryl groupings of the muscle proteins produce a negative oxidation-reduction potential. Further the solid fragments when packed together minimise the entrance of oxygen into the medium. Thus even asbestos wool has been found effective in making a fluid medium suitable for growth of anaerobes.

The Use of Hydrogen for Anaerobic Cultures.—The gas is generated in a large Kipp's apparatus from pure 25 per cent. sulphuric acid and pure zinc. It is passed through three wash-bottles. In the

[1] Supplied by the Viscose Development Co. Ltd., Woldham Road, Bromley, Kent.

first is placed a solution of lead acetate (1 in 10 of water) to remove any traces of sulphuretted hydrogen. In the second is placed a 1 in 10 solution of silver nitrate to remove any arseniuretted hydrogen which may be present if the zinc is not quite pure. In the third is a 10 per cent. solution of pyrogallic acid in caustic potash solution (1 in 10) to remove any traces of oxygen. The tube leading from the last bottle to the vessel containing the medium ought to be sterilised by passing through a Bunsen flame, and should have a small plug of cotton wool in it to filter the hydrogen germ-free.

Commercial hydrogen as sold in cylinders may be used; it is necessary, however, to reduce the pressure, and this can be done

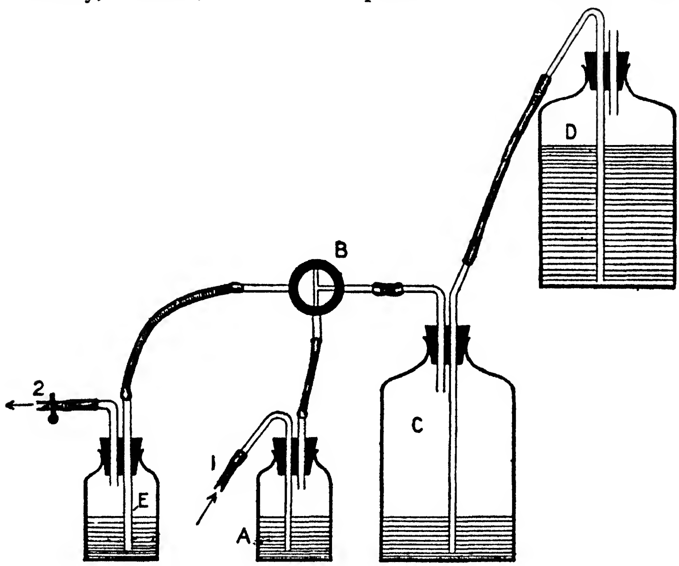


Fig. 15.—Arrangement of bottles for reducing pressure in hydrogen supply from cylinder. (After Mackie and McCartney.)

as follows (Fig. 15). The gas from the cylinder is passed by tube (1), not too rapidly, through the wash-bottle A; from this it passes by the three-way stop-cock B to a jar C of 15–20 litres capacity which is graduated roughly in litres. The jar C is furnished with an indiarubber stopper and tubing as shown. The hydrogen entering C forces the water into a similar jar D which is supported 4 feet above C. When sufficient gas has entered C the supply valve from the cylinder is closed. The stop-cock B is then turned, so that the hydrogen passes from C through the wash-bottle E, and passes by tube (2) to the anaerobic jar, the flow being adjusted so as to produce a not too rapid stream of bubbles. As the gas escapes from C it is replaced by water from D. All stoppers must be air-tight; this can be secured by means of sealing wax. All the rubber tubing must be of the thick-walled "pressure" type.

An alternative method is to attach a *reducing valve* to the gas-cylinder which will deliver the hydrogen at a constant pressure, *e g.* of 2 or 3 lb. per sq. in., as indicated by a gauge connected to the delivery tube. The flow of hydrogen into the anaerobic jar can be observed by interposing a small wash-bottle between the valve and the jar, similar to that shown in Fig. 15.

The Use of Pyrogallic Acid and Caustic Soda.—This depends on the fact that sodium pyrogallate rapidly absorbs oxygen from the air (becoming dark brown in colour), thus the air in an enclosed space can be deprived of its oxygen. For the absorption of oxygen from a container of 4 litres capacity, the amounts used are 109 grams solid caustic soda dissolved in 145 c.c. water, to which 4 grams pyrogallol are added. Various forms of apparatus have been used.

Buchner's Tube is applicable in the case of sloped cultures in test-tubes. This consists of a tube of thick glass measuring about $8\frac{1}{2} \times 1$ inch, with the lower end constricted, so that the culture tube does not reach the foot. The Buchner's tube is provided with a tightly fitting india-rubber stopper. Solid caustic soda (three pieces each of about the size of a pea—2 grams) is placed in the bottom of the tube and covered with several layers of filter-paper ; about 15 c.c. 10 per cent. solution of pyrogallic acid are added ; then the

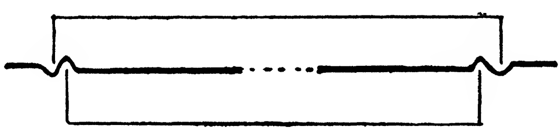


Fig. 16.—Henry's apparatus.

inoculated culture tube is quickly introduced and the tube is closed with the stopper.

Another method consists in inoculating an agar slope in a thick-walled test tube, cutting off the free end of the absorbent cotton-wool stopper till about 2 cm. remains, pushing the stopper into the tube for 2 cm. and filling the space with NaOH granules, then pouring in 4–5 c.c. 10 per cent. pyrogallic acid solution. The tube is finally closed with a well-fitting india-rubber stopper, inverted so that water of condensation does not run over the agar surface, and incubated in this position.

Henry's Method.—In this modification two shallow circular dishes (portions of Petri capsules) are separated by a tin diaphragm, in the centre of which is an aperture (Fig. 16). The upper dish contains the plate culture, the lower (smaller) contains 2 grams of caustic soda granules. Grooves are present in the metal to receive the margins of the dishes, which are fixed in with plasticine. The lower dish is first fixed in position, and just before the upper dish is adjusted, 10 c.c. of 10 per cent. pyrogallic acid solution are run into the lower through the opening in the plate.

McIntosh and Fildes' Anaerobic Jar.—These authors have designed a jar in which tubes may be incubated under anaerobic conditions, the oxygen being absorbed by spongy palladium. A glass jar is employed furnished with a metal lid which can be clamped

down. The lid is fitted with a tube and valves so that hydrogen may be admitted into the jar. In the improved form of the apparatus the palladium asbestos is fixed on an insulated spool and surrounded by a piece of resistance wire which is connected to two electric terminals on the outside of the lid. Cultures are placed inside the jar and hydrogen is passed through the jar for half a minute. Then the valves are closed and the terminals are attached to the electric supply, which must be reduced by suitable resistances. As a result of the consumption of oxygen by the hot palladium asbestos the pressure within the jar falls, and after the current has been passing for ten minutes more hydrogen may be added at very low pressure; the current should then be allowed to pass for half an hour. Along with the cultures an indicator should be placed in the jar. This consists of a mixture in a test-tube of equal volumes

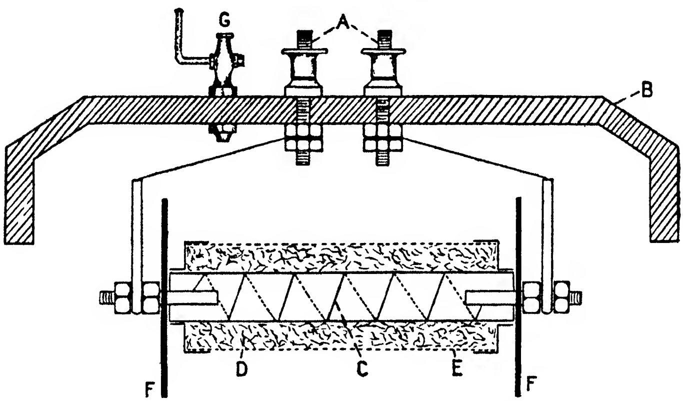


FIG. 17.—Top of anaerobic jar. (Fildes and McIntosh.)

A. Electric terminals. B. Section of metal top of jar. C. Coil of wire around insulator. D. Asbestos E. Gauze cover. F. Mica plates. G. Gas inlet.

of (*a*) N/10 NaOH 6 c.c., water to 100 c.c., (*b*) 3 c.c. ½ per cent. watery methylene blue, water to 100 c.c., (*c*) glucose 6 grams, water to 100 c.c., and a small crystal of thymol; this is boiled till reduced and at once placed in the jar. This indicator, when in the jar, should remain colourless except for a slight tinge of blue at the top, which slowly disappears during the passing of the current. To secure very thorough anaerobiosis, current may be passed at intervals during the period of incubation. It is absolutely necessary that the lid of the jar should be air-tight. This can be tested by placing a few drops of ether in the jar, fixing on the lid, and plunging the vessel in hot water; any leak can thereby be detected. To avoid danger from accidental explosion, glass jars should be covered with a cloth and placed in a wooden box. Metal jars are obtainable also.

Absorption of Oxygen by Bacterial Growth.—This method, recommended by Fortner, depends on the fact that certain organisms

when growing in a confined space actively remove the oxygen in the contained air. A ring of aluminium one-ninth of an inch thick and with an aperture of 3 inches is made to fit closely into the lid of a 4-inch Petri capsule. Melted agar is poured into the space in the ring and, when set and dry, its surface is inoculated with a suitable organism (*B. coli, B. pyocyaneus, B. prodigiosus*, etc.). Glucose agar is poured into the other half of the capsule and is inoculated with the anaerobic organism. Then the two parts are closed with plasticine. In order to avoid contamination from "creeping" of the culture on to the other half of the plate through condensation of moisture, the plate, except for momentary inspection, should not be removed from the incubator without at once separating the two halves.

Isolation of Anaerobes.—Shake cultures may be made in a series of deep tubes of glucose agar which have been melted and cooled to 45° C. The inoculum is mixed with the agar by rolling the tube, which is then allowed to solidify and is incubated as usual. To make subcultures from separate colonies a capillary pipette (made by drawing out a piece of quill tube) sterilised by heat is passed down to the colony and some of the growth is sucked up into it. This method, however, is often unsatisfactory owing to the tearing of the medium by gas-formation. Accordingly recourse must be had to surface cultures. The obtaining of pure cultures from mixtures of anaerobes usually presents very great difficulty, and repeated subculturing from isolated colonies is necessary. Various other devices may have to be employed. A mixed culture in cooked meat medium may show preponderance of one organism at a particular period of growth, when subcultures should be made. Non-sporing contaminants can readily be excluded by exposing a suspension of the organisms to 80° C. for half an hour. Differential susceptibility of the spores to heat may enable one species to be killed while the more resistant survives. Again, in surface growths certain organisms, *e.g. B. tetani*, tend to spread out farther than others and so to be found pure at the margins. By means of animal inoculation pathogenic members may be recovered from mixtures with non-pathogens; or where several pathogenic types are present, the wanted organism may be permitted to flourish alone in the body of an animal passively immunised beforehand by an injection of antiserum to the other species in the mixture.

The Incubation and Storing of Cultures.—Gelatin cultures must be grown at a temperature below their melting-point, *i.e.* for 10 per cent. gelatin, below 22° C. They are usually kept in ordinary rooms or in a cool incubator at about 20° C. Agar and serum media are employed to grow bacteria at a higher

temperature, corresponding to that at which the organisms grow best, usually 37° C. in the case of pathogenic organisms. For the purpose of maintaining a uniform temperature incubators are used. These vary much in the details of their structure, but all consist of a chamber provided with a source of heat regulated by a thermostat. Patterns in which gas or oil is used have double walls between which fluid (usually water) is placed. Electrically heated incubators, without water ("anhydric"), are very convenient. Where large quantities of cultures are dealt with a warm room is advantageous; the temperature of this is regulated similarly to that of an incubator, but to prevent undue cooling on entering the room, it is provided with double doors the space between which acts as an air-lock, so that the outer door is closed before the inner is opened and *vice versa*

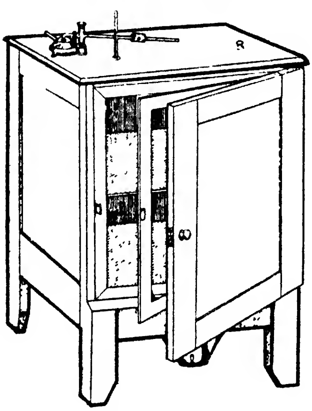


Fig. 18.—Hearson's incubator for use at 37° C. (form heated by gas).

It is necessary to prevent too much evaporation from the surface of cultures placed within incubators, otherwise they may quickly dry up. With tubes which will require to be long in the incubator, the plugs should be pushed a little way into the tube and a few drops of melted paraffin dropped on the top of the wool, or the plugs should be covered by indiarubber caps previously sterilised in 1 : 1000 corrosive sublimate and then dried. Before they are placed on the tubes the cotton-wool plugs ought to be well singed in a flame. Cultures in bottles with screw-caps are also protected from evaporation. Another method of preventing evaporation, which is useful for the maintenance of stock cultures, is to keep them as stab cultures. Generally for preserving the life of bacteria in cultures the medium should not contain a fermentable sugar as the acid formed is harmful. Also, media which do not yield a very abundant growth often maintain life better than rich media. Special methods of preserving cultures are thorough desiccation or maintenance at a temperature below freezing-point or a combination of these.

Filtration of Cultures.—For many purposes it is necessary to filter all the organisms from fluids in which they may have been growing. This is done especially in obtaining the soluble toxic products of bacteria, and also when investigating "ultra-microscopic" or "filter-passing" viruses. Filters capable

of keeping back such minute bodies as bacteria consist of a tube of unglazed earthenware as introduced by Chamberland. The efficiency of such a filter depends on the fineness of the grain of the clay from which it is made. The porosity of the Pasteur-Chamberland (French) filters varies and is specified by the makers. L 1–3 grades are of increasing fineness but the last may allow some bacteria to pass. L 5–13 grades are stated to retain all the ordinary bacteria. Doulton (British) and Maassen (German) filters are similar to the above. There are several other types of filters, differing in character, *e.g.* the Berkefeld[1] filter made of diatomaceous earth and the Mandler (American). In the Seitz (E. K.) asbestos filter, a disc of fibrous material is used as the filtering agent; this form has the advantage that the discs are readily replaceable. For the study of viruses by ultrafiltration, extremely fine-pored filters are obtained by the use of collodion membranes.

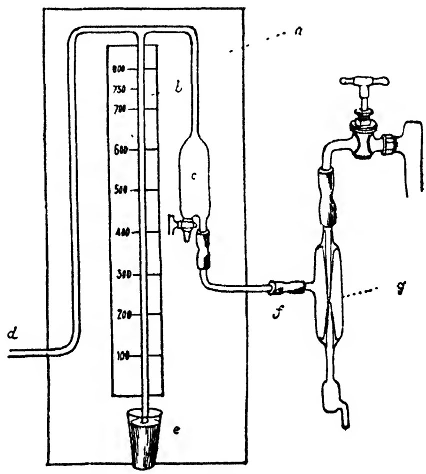


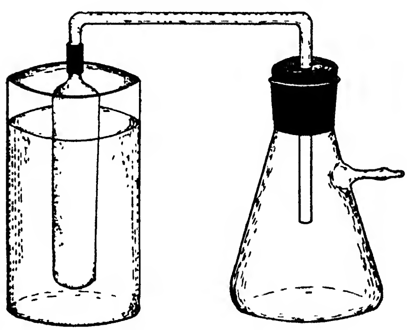
FIG. 19.—Geissler's vacuum pump arranged with manometer for filtering cultures. (The tap and pump are intentionally drawn to a larger scale than the manometer board to show details.)

For ordinary bacteriological work, the most generally convenient filtering apparatus is that in which the fluid is sucked through the porcelain by exhausting the air in the receptacle into which it is to flow. This is conveniently done by means of a Geissler's water-exhaust pump (Fig. 19, *g*), which must be securely fixed to a tap leading directly from the main, all connecting tubes being of heavy indiarubber. A manometer tube (*b*) and a receptacle (*c*) (the latter to catch any back flow of water from the pump which may accidentally occur) are intercepted between the filter and the pump. These are usually

[1] Berkefeld filters are made of three grades of porosity, designated V, N and W in diminishing order.

arranged on a board *a*. Between the tube *f* and the pump *g*, and between the tube *d* and the filter, it is convenient to insert lengths of flexible lead-tubing connected up at each end with short, stout-walled rubber-tubing. An air-pump, *e.g.* of Geryk type, may be used instead.

Filters are arranged in various ways. (*a*) An apparatus is arranged as in Fig. 20. The fluid to be filtered is placed in the cylindrical vessel *a*. Into this a "candle" or "bougie" of porcelain dips. From the upper end of the bougie a glass tube with thick rubber connections proceeds to flask *b*. From a side arm of the flask a similar tube proceeds to the exhaust-pump. When the latter is put into action the fluid is sucked through the porcelain and passes over into flask *b*. This apparatus is not suitable for small quantities of fluid.



a *b*

FIG. 20.—Chamberland candle and flask arranged for filtration.

(*b*) A very good apparatus can be arranged with a lamp funnel and the porcelain bougie. The open end of the filter candle is glazed so as to render it impermeable. A tightly fitting perforated indiarubber bung is slipped over this part of the filter; the lower part of the bung fits into the neck of the flask, the upper part fits into a glass cylinder (Fig. 21*a*). The efficiency of such a filter, especially when small amounts of fluid are being dealt with, is much increased if when the level of the fluid falls below the upper end of the candle a closely fitting test-tube is slipped over the latter. By this device the leakage of air through the exposed part of the candle is prevented.

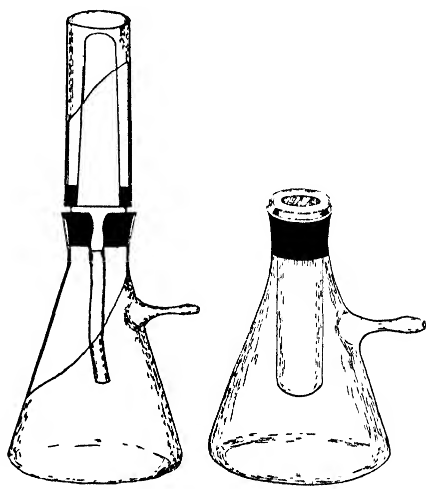
(*c*) A further arrangement is shown in Fig. 21*b*. It is very suitable for a type of filter provided with a wide glazed flange at the mouth. The material to be filtered is placed inside the candle; small amounts can be filtered in this way.

(*d*) The Seitz disc is inserted into a holder.[1] It is very effec-

[1] A form of this filter in which the holder is made of glass and stainless steel, is supplied by Messrs. A. Gallenkamp, 17 Sun Street, London, E.C.2.

tive and convenient in use. A fresh disc is employed for each filtration.

Before any one of the above apparatus is used it ought to be connected up as far as possible and sterilised in the autoclave or Koch's steriliser. The ends of any important unconnected parts ought to have pieces of cotton wool tied over them. After use the bougie is to be sterilised in the autoclave or by soaking in antiseptic solution. Much of the material kept back on the filter can now be removed by forcing water through in a direction opposite to that of the flow of the fluid during filtration. Colloidal matter tends, however, to be retained in the pores of the filter and to alter its properties. This may be dissolved by treatment with antiformin, then passing dilute HCl through the filter and finally water, until the filtrate reacts neutral. Alternatively, the candle, after being dried, may be carefully burned in a muffle furnace till the original colour is regained.



a *b*

Fig. 21.—Other arrangements of filtering candle and flask.

The material to be filtered should be freed from gross particles by centrifuging or filtration through paper. Highly viscous fluids, *e.g.* those containing much protein, filter slowly unless diluted with water or saline; warming up to 37° C. may also facilitate filtration in such cases. The success of filtration must be tested by inoculating media with considerable amounts of the filtrate, and observing if growth takes place, as there may be minute perforations in a candle sufficient to allow bacteria to pass through. It is often advisable to add to the fluid immediately before filtration a suspension of an agar culture of an easily identifiable organism such as *Staphylococcus aureus* or *B. prodigiosus*; or a suspension of one of these organisms in the same fluid as that filtered and in similar amount may be

passed through the filter immediately after the main experiment.

In all critical work involving filtration, it is necessary to note the area of the filtering surface, the amount of filtrate, the pressure at which filtration took place and the duration of the process.

Other aspects of filtration are discussed later (p. 715).

Clarification is an essential preliminary to filtration where gross particulate matter is present, *e.g.* in tissue suspensions which are to be investigated for viruses. This may be effected by centrifuging, passing through a column of paper pulp or a Jena "sintered" glass filter. The paper pulp clarifier is made by placing a glass wool plug in a cylindrical filter funnel and superimposing in order, 1 cm. of silver sand, 2 cm. of paper pulp (made by working up compressed neutral-reacting paper pulp to an even mass in hot water) and 1 cm. of silver sand—the whole being sterilised by autoclaving before assembling.

Centrifuges.—For many purposes of bacteriological investigation a centrifuge, with a speed up to 4000 revolutions per minute, is essential. It should be capable of holding four tubes each of 15–30 c.c. capacity; the motive power may be water or electricity—a hand-driven centrifuge may be used. For separating bacteria from large volumes of fluid centrifugal separators are useful.[1] For the investigation of viruses high speeds are essential, *e.g.* up to 50,000 revolutions per minute.[2]

The Drying of Substances *in vacuo*.—As many substances, for example toxins and antitoxins, with which bacteriology is concerned would be destroyed in drying by heat as is done in ordinary chemical work, it is necessary to remove the water at the ordinary room temperature. This is most quickly effected by drying *in vacuo* in the presence of some substance, such as strong sulphuric acid, calcium chloride or phosphorus pentoxide, which readily takes up water vapour. The vacuum produced by a water-pump is here not suitable, as in such a vacuum there must always be water vapour present. An air-pump is therefore to be employed. The Geryk pump is very efficient for the purpose.[3]

Refrigeration.—Some form of refrigerator which will maintain a temperature of $-10°$ to $-15°$ C. is of the greatest

[1] A serviceable form is made by the Alfa-Laval Co., Brentford, Middlesex, *e.g.* size 100.

[2] A machine of this kind on the principle of Henriot and Huguenard, modified by McIntosh, is supplied by Baird and Tatlock, 14 Cross Street, Hatton Garden, London, E.C.1.

[3] For luting glass-stoppers, etc., Stevens' rubber grease is effective.

value for preserving antisera (to which antiseptics should not be added) and other biological products. Many organisms can be kept alive for long periods in the frozen state.

Method of Mounting Bacterial Cultures as Permanent Museum Specimens (Richard Muir).—(*a*) *Stab or Stroke Cultures in Nutrient Gelatin or Agar Media.*—When the culture shows typical characters, further growth is arrested by placing the tube in a formol vapour chamber, or by saturating the cotton-wool plug with strong formalin. Then leave for a day or two. Make up the following :

(1)	Thymol water (saturated in cold) . . .	100 c.c.
	Glycerol	20 ,,
	Acetate of potash	5 grams.
	Coignet's (gold label) gelatin . . .	10 ,,

Render the mixture acid to litmus with acetic acid ; clear with white of egg, and filter.

Warm to about 40° C., and, removing cotton-wool plug from culture, take a little of the preserving fluid in a pipette and allow to run gently over surface of medium in tube. Place in such a position that a thin layer of the preserving medium remains completely covering the growth and the surface of culture medium. The gelatin is now allowed to solidify. Add three or four drops of strong formalin to the tube, and fill up to within a quarter of an inch of the top of the tube with the following fluid :

(2)	Thymol water (saturated in cold) . .	100 c.c.
	Glycerol	20 ,,
	Acetate of potash	5 grams.

Cover top of tube with a small piece of paper so as to keep out dust, allow to stand for a day or two so that small air-bells may rise to the surface.

To seal tube, pour melted paraffin gently on to the surface of fluid up to near the top of tube ; allow to solidify. Cover paraffin with layer of alcoholic orange shellac cement ; allow this to set, and repeat until the cement becomes level with top of test-tube. When the cement is set, a few drops of black lacquer are put on, and a circular cover-glass of about the same diameter as the mouth of tube is placed so as completely to seal it.

(*b*) The following method is useful for preserving *plate cultures* : Instead of making the cultures in Petri's capsules, use ordinary watch-glasses. The watch-glass is sterilised in a Petri's capsule, and the inoculated medium is poured out into the watch-glass, allowed to solidify in the usual way, and left in the Petri's capsule until the colonies of growth have developed. The watch-glass is now removed from the capsule, and a layer of the preserving gelatin medium (1) to which have been added a few drops of strong formalin, is allowed to spread over the surface of the culture medium. When the layer is solidified the watch-glass is filled up with the same, and a clean square or oblong piece of glass (which, of course, should be slightly larger than the watch-glass diameter) is now carefully placed over watch-glass, care being taken that no air-bells are formed. The edge of the watch-glass should be closely applied

to the glass cover, and left in position until the gelatin has solidified. The superfluous gelatin is now removed, and the glasses sealed first with the orange shellac cement, then with black lacquer. It is now finished off by using a circular mask of suitable size.

The various kinds of solid media used in the cultivation of bacteria, such as blood serum, potato, etc., can be treated in the same manner with excellent results.

General Laboratory Rules.—On the working bench there should be a large dish of 1 : 1000 solution of mercuric chloride in water, or 10 per cent. liquor cresolis saponatus. Into this all discarded tubes, vessels, plates, hanging-drop cultures, etc., which have contained non-sporing bacteria ought to be at once placed (in the case of tubes, the tube and plug should be put in separately). On no account whatever are such infected articles to be left lying about the laboratory. The basin is to be repeatedly cleaned out. All the glass is carefully washed in repeated changes of tap water to remove the last trace of perchloride of mercury, a very minute quantity of which is sufficient to inhibit growth. Old cultures which have been stored for a time, and from which fresh subcultures have been made, ought to be steamed in the Koch's steriliser for two or three hours, or should be autoclaved, and the tubes thoroughly washed out. Besides a basin of antiseptic solution for infected apparatus, etc., there ought to be a second reserved for the worker's hands in case of any accidental contamination. When a large number of tubes are being daily put out of use, they may be placed in an enamelled iron pail, and this when full is placed in the steam steriliser. Cultures and other materials containing sporing organisms, *e.g. B. anthracis* or *B. tetani*, ought to be autoclaved. Pipettes and other glass ware contaminated with spores of pathogens may be immersed in concentrated sulphuric acid. It should be noted that bacteria in films prepared and stained in the usual way with simple watery stains may still be alive; accordingly, suitable precautions should be taken in handling such preparations.

A white glazed tile on which a bell-jar can be set is very convenient to have on a bench. Infective material in watch-glasses can be placed thus under cover, and if anything is spilled the whole can be easily disinfected. In examining organs containing virulent bacteria, these should not be touched with the bare hands. No food ought to be partaken of in the laboratory, and pipes, etc., are not to be laid with their mouthpieces on the bench. No label is to be licked with the tongue. Before leaving the laboratory the worker ought

to wash the hands and forearms with antiseptic solution and then with yellow soap. In the case of any fluid containing bacteria being accidentally spilt on the bench, etc., 10 per cent. liq. cresol. sap. is to be at once poured on the spot. The air of the laboratory ought to be kept as quiet as possible.

CHAPTER III

MICROSCOPIC METHODS

The Microscope—For ordinary bacteriological work a good microscope is essential. It ought to have a heavy stand, with coarse and fine adjustments, a double mirror (flat on one side, concave on the other), a good condenser, with an iris diaphragm, and a triple nose-piece. Three achromatic objectives are necessary, dry low and high powers ($\frac{2}{3}$ and $\frac{1}{6}$ inch) and a $\frac{1}{12}$-inch oil immersion (N.A. 1·3). It is preferable to have two eyepieces ($\times 5$ and $\times 10$), but a single one $\times 8$ will serve. The flat side of the mirror ought to be used along with the condenser. The condenser should be at such a height that the rays of light from the illuminant are brought to a focus in the plane of the preparation to be examined; as a rule, this result is attained approximately when the upper surface of the condenser is practically on the same level as the stage of the microscope. To adjust the condenser accurately, place a microscopic preparation on the stage and focus it with the low-power objective. Then, keeping the position of the objective unchanged, rack the condenser till an image of the lamp is seen sharply; if daylight is used, focus a part of the window frame. The condenser is then almost at the proper level for use with the immersion lens.

The oil immersion lens is used as follows. After the light is satisfactorily arranged, fix the slide on the stage with the right-hand clip and place a drop of cedar-wood oil on the preparation; lower the objective till the point touches the drop of oil. Then, with the eye at the ocular, rack the tube of the microscope slowly down till the preparation comes into focus; then focus with the fine adjustment. In the case of stained bacteria, when a pure coloured picture is desired, as in examining stained films, the diaphragm ought to be *widely opened*. If the microscope is not fitted with a movable stage, it is convenient to use the left hand for moving the preparation whilst the right operates the fine adjustment. When the observer has finished for the time being with the immersion lens he ought to wipe off the oil with a piece of silk or very fine washed linen. If the oil has dried on the lens it may be moistened with xylol—never with alcohol,

which will damage the material by which the lens is fixed in its metal carrier.

Microscopic Examination of Bacteria.—1. Hanging-drop Preparations.—Micro-organisms may be examined: (1) alive or dead in fluids; (2) in film preparations; (3) in sections of tissues. In the two last cases advantage is always taken of the affinity of bacteria for certain stains. When they are to be examined in fluids[1] (p. 85), it is necessary to use a small aperture of the condenser-diaphragm. It is best to focus the edge of the drop with a low-power objective, and, the slide being arranged so that part of the edge crosses the centre of the field, to fix the preparation in this position with a clip. A high-power lens is then turned into position, and lowered by the coarse adjustment to a short distance above its focal distance; it is now carefully screwed down by the fine adjustment, the eye being kept at the ocular meanwhile. The shadow of the edge will be first recognised, and then the bacteria must be carefully looked for. Often a dry lens is sufficient, but for some purposes the oil immersion is required. If the bacteria are small and motile, a beginner may have great difficulty in seeing them, and it is well to practise at first on some large non-motile form, such as *B. anthracis*. In fluid preparations the natural appearance of bacteria may be studied, and their rate of growth determined. The great use of such preparations, however, is to find whether or not the bacteria are motile, and for determining this point it is advisable to use either broth or agar cultures not more than twenty-four hours old, or preferably younger; often motility is more readily detected in cultures grown at room temperature than at 37° C. In the case of cultures on solid medium, a small fragment of growth is broken down in broth or in sterile water. In determining whether or not a bacterium is motile, great difficulty is often experienced in distinguishing between true motion and Brownian movement, especially if the organism be small. The essential criterion to be fulfilled is that the bacteria shall be moving in all directions, the observation of individuals close together starting to move in opposite directions being important. The observation of hanging-drop preparations must be correlated with the results of staining for the presence of flagella which, so far as is known, are present in all motile forms except spirochætes.

Dark-Ground Illumination.—The principle of this method is

[1] In bacteriological work it is essential that cover-glasses of No. 1 thickness (*i.e.* 0·14 mm. thick) should be used, as those of greater thickness are not suitable for a $\frac{1}{12}$-inch lens.

that the object to be examined is illuminated by rays of light brought to a focus on the object obliquely, no direct rays from the source of illumination reaching the eye of the observer. The object is thus seen brightly lighted on a dark background. A special form of condenser is required, which may be of the spherical or paraboloid type. In the former, the central rays of the beam of light are interrupted by a circular diaphragm while the lateral rays are reflected outwards from the lower

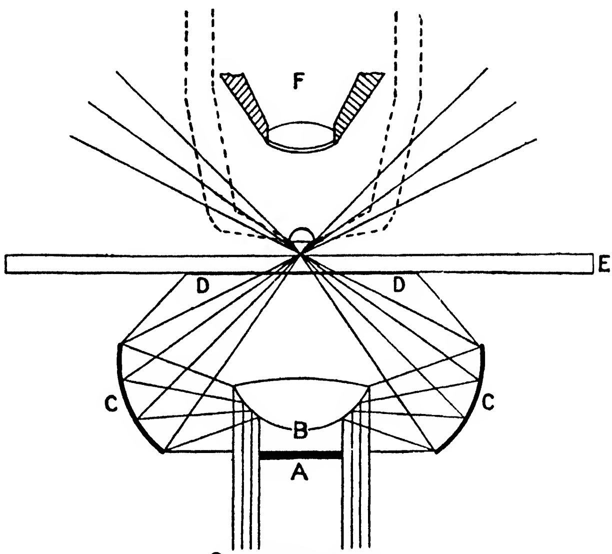


FIG. 22.—Diagram showing course of rays in dark-ground illumination.
A. Circular stop. B and C Reflecting surfaces. D Drop of oil between condenser and slide. E. Microscopic slide F. Lens

surface of a spherical reflector and then inwards from another reflecting surface, and are brought to a focus in the position of the object to be examined (Fig. 22). Within the oil immersion lens to be used a stop is placed so as to cut down its N.A. to 1 or less. The source of light is a small arc lamp or an " Osram " 100 watt projection lamp, with a condensing lens which forms a beam of approximately parallel rays. The following are the steps in the procedure :

(1) The microscope with special condenser is placed in front of the source of light so that the beam of light occupies the surface of

the substage mirror, the flat surface being used and the mirror being placed at an angle to direct the vertical rays of light upwards in the optical axis. (2) Centre the illumination. To do this use a low-power ocular; remove the condenser and swing out the objective. Then, holding a card or sheet of paper above the ocular, at right angles to the axis of the microscope tube, adjust the mirror so that a uniform disc of light is seen on the paper. (3) Replace the condenser and rack it up to the level of the stage. Place a drop of oil free from bubbles on the upper surface of the condenser; then rack the latter down a little. Place a drop of oil on the under surface of the slide just beneath the preparation to be examined; place the latter on the stage and fix it with the clips. Then rack the condenser slowly up till the oil spreads in a uniform layer between the upper surface of the condenser and the slide (Fig. 22). (4) Focus the preparation with a low-power objective. Rack the condenser a little till a bright spot of light is seen and then bring this by means of the adjusting screws of the condenser into the middle of the field. (5) Put a drop of oil on the cover-glass of the preparation. Place the immersion lens (fitted with the stop as described) in position and focus in the usual way. A little adjustment of the mirror or of the centring screws may be necessary to get the optimum result—brilliantly lighted objects on a black background.

(In some patterns of condenser concentric rings are engraved on the upper surface; centring may be effected in such a case *before stage* (3), as follows: replace the condenser and rack it up to the level of the stage; focus the upper surface of the condenser with a low power objective when the rings will come into view, and adjust the centring screws of the condenser until the rings are concentric with the field. Then place a drop of oil on the surface of the condenser and proceed as in stages (3) and (5).)

It is a great advantage to use a separate microscope for dark-ground work and to have it mounted on a wooden stand along with the electric lamp and the condensing lens.

NOTE.—Slides to be used for the dark-ground method ought not to exceed a thickness which is specified by the makers of the condenser (usually 1·2 mm.); if thicker, the light will be brought to a focus below the level of the film.

2. **Film Preparations.**—(*A*) *Dry Method.*—This is the most extensively applicable method for microscopically examining bacteria. Fluids containing bacteria, such as blood, pus, scrapings of organs, can be thus investigated, as also cultures in fluid and solid media. For all ordinary purposes films are made on slides, but sometimes cover-glasses are used. The first requisite is a perfectly clean slide or cover-glass. The test of this is that, when the drop of fluid containing the bacteria is placed upon the glass, it can be uniformly spread with the platinum wire all over the surface without showing any tendency to retract into droplets. The best method is to place the cover-glasses or slides for some time in a mixture of concentrated nitric acid 6 parts, potassium bichromate 6 parts,

water 100 parts. After thorough washing in water they may be kept in 50 per cent. alcohol; for use, they are dried with a soft clean cloth. Slides may be rapidly cleaned by rubbing the surface with a moist cloth which has first been rubbed on a fine sand soap, then washing quickly under the tap and drying with a clean rag—this method must not be used with slides intended for dark-ground examination. If a fluid is to be examined, a loopful may be placed on the glass and spread out over the surface with the wire. When a culture on a solid medium is to be examined, a loopful of water is placed on the slide, and a minute particle of growth rubbed up in it and spread out, so as to form a thin film. The great mistake made by beginners is to take too much of the growth. The point of the straight wire should just touch the surface of the culture, and when this is rubbed up in the droplet of water and the film dried, there should be an opaque cloud just visible on the slide. In the case of *pus* or *sputum* a loopful should be spread out on the slide so as to make streaks of varying thickness. When a film has been spread, it must next be dried at room temperature or by being waved backwards and forwards at arm's-length above a Bunsen flame. The film must then be fixed on the glass; in the case of a slide, the under side is heated in the Bunsen flame until it is just too hot to be borne on the palm of the hand; charring must be avoided. When cover-glasses are used for films, Cornet's forceps will be found convenient for handling them (Fig. 23); fixation is effected by passing the cover-slip two or three times through the flame.

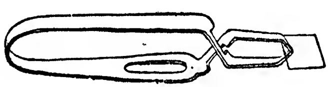


FIG. 23.—Cornet's forceps for holding cover-glasses.

In the case of *blood,* a small drop is placed near one end of a clean slide, the edge of a second slide is lowered through the drop on to the surface of the glass on which the blood has been placed. This second slide is held at an angle to the first, and the droplet of blood by capillarity spreads itself in the angle between the two slides. The edge of the second slide is then pushed slowly along the surface of the first slide, and the blood is spread out in a film whose thickness can be regulated by the angle formed by the second slide. Large-sized films can thus be obtained. A film prepared in this way may be too thick at one edge, but at the other is sufficiently thin. Another method is to allow a drop of blood to spread itself between two clean cover-glasses, which are then to be slipped apart, and being

held between the forefinger and thumb are to be dried by a rapid to-and-fro movement in the air. If it is desired to preserve the red blood corpuscles in a film, it may be fixed by one of the following methods: by being placed (*a*) in a hot-air chamber at 120° C. for half an hour; (*b*) in methyl alcohol for two to three minutes, then washed and dried; (*c*) in formol-alcohol (Gulland)—formalin 1 part, absolute alcohol 9 parts—for five minutes, then washed and dried. In using the Romanowsky stains no previous fixation is necessary (*vide infra*).

In the case of *urine*, the specimen is centrifuged and films are made from the deposit. After dried films are thus made from urine it is an advantage to place a drop of distilled water on the film and heat gently to dissolve the deposit of salts; then gently wash in water and dry. In this way a much clearer picture is obtained when the preparation is stained.

Films dried and fixed by the above methods are now ready to be stained by the methods to be described below.

(*B*) *Wet Method.*—If it is desired to examine the histological structure of the cells of a discharge as well as to investigate the bacteria present, it is advisable to substitute "wet" films for the "dried" films, the preparation of which has been described. The nuclear structure, mitotic figures, etc., are by this method well preserved, whereas these are considerably distorted in dried films. The initial stages in the preparation of wet films are the same as above, but instead of being dried in air they are placed, while still wet, film downwards in the fixative. The following are some of the best fixing fluids:

(*a*) Saturated solution of perchloride of mercury in water. Fix for five minutes. Then rinse the films in water and thereafter wash in successive strengths of methylated spirit; treat with Gram's iodine to remove excess of mercuric salt, and again with spirit. After this treatment the films are stained and treated as if they were sections

(*b*) Formol-alcohol—formalin 1 part, absolute alcohol 9. Fix films for three minutes; then wash well in methylated spirit. They are then ready for staining. This is an excellent and very rapid method.

(*c*) Corrosive-alcohol—alcohol 1 part, saturated solution of mercury perchloride 2 parts. Fix for five minutes, wash with 50 per cent. spirit, and treat as in (*a*). This fixative is very suitable for films of fæces containing entamœbæ (p. 822).

3. **Examination of Bacteria in Sections.**—The methods of fixing and cutting tissues will be described below. Paraffin sections give by far the best results, and we shall suppose that the sections have been fixed on slides by the method given (p. 134). The procedure is then as follows: Several drops of xylol are placed on the section and made to move backwards and forwards; it is poured off and the process is repeated. The excess of xylol

is then removed by a clean cloth, care being taken not to allow the preparation to dry. A few drops of absolute alcohol are allowed to run through the preparation so as to remove the xylol. This is followed in the same way by methylated spirit, and the slide is then placed in a bowl of water. The section is then ready for staining. In the following account we shall assume that the sections have all been brought to this stage. Sections cut on the freezing microtome are also brought into water and are then to be similarly treated.

Sections after being stained are (*a*) dehydrated, (*b*) cleared, and (*c*) mounted in Canada balsam. Dehydration is usually effected by alcohol. The preparation is dried with blotting-paper and treated with a few drops of alcohol, then cleared in xylol, and mounted in a drop of balsam. After being treated with xylol the preparation ought to be perfectly clear ; if any opacity remains, further dehydration with alcohol is necessary. For certain purposes, *e.g.* when the bacteria are readily decolorised by alcohol, aniline-xylol (aniline 2 parts, xylol 1 part) is used as the dehydrating agent. The preparation is blotted as before and treated with aniline-xylol which clears at the same time. The aniline-xylol is then replaced by xylol ; this is conveniently done by running the xylol through the preparation in one direction, when the aniline-xylol will be seen to be displaced before it. The preparation is then mounted in balsam.

The Staining of Bacteria

Staining Principles.—To speak generally, the protoplasm of bacteria reacts to stains in a manner similar to the nuclear chromatin, though sometimes more and sometimes less actively. The bacterial stains *par excellence* are the basic aniline dyes, which have the constitution of salts. Aniline dyes are divided into two groups according as the staining action depends on the basic or the acid portion of the molecule. Thus the acetate of rosaniline derives its staining action from the rosaniline ; it is therefore called a basic dye. On the other hand, ammonium picrate owes its action to the picric acid part of the molecule ; it is therefore termed an acid dye. These two groups have affinities for different parts of the animal cell. The basic stains have a special affinity for the nuclear chromatin, the acid for the cytoplasm and various formed elements. Thus it is that the former—the basic aniline dyes—are especially the bacterial stains ; but certain acid dyes, *e.g.* eosin or rose bengal, also stain bacteria.

The following are the most commonly used basic stains (the figures in brackets after each indicate the number in the Colour Index (C.I) of the Society of Dyers and Colourists [1] and the approximate percentages soluble in 95 per cent. alcohol and in water respectively) :

Violet Stains.—Crystal violet, chloride (C I. No. 681 ; 14, 1 7) and the closely related methyl-violet (C I. No. 680 ; 15, 3) and gentian-violet.

Blue Stains.—Methylene-blue, chloride (C.I. No. 922 ; 1·5, 3·6) (synonym : phenylene-blue) ; Thionin (C.I. No. 920 ; 0·25, 0·25) (also known as Lauth's violet) ; Toluidine-blue (C.I. No. 925 ; 0·57, 3·8).

Red Stains.—Basic fuchsin, chloride (C.I. No. 677 ; 8, 0·4) (synonyms : basic rubin, magenta, rosanilin) ; Safranin (C.I. No. 841 ; 3·4, 5·5) (synonyms : fuchsia, giroflé).

Brown Stains —Bismarck-brown (C.I No. 331 ; 1, 1·4) (synonyms · vesuvin, phenylene-brown) ; Chrysoidin (C.I. No. 20 ; 2·2, 0·86).

Of the stains specified, the violets and reds are the most intense in action, especially the former ; it is thus easy in using them to over-stain a specimen. It is difficult to over-stain with methylene-blue. Thionin also gives good differentiation and does not readily over-stain.

It is most convenient to keep saturated alcoholic solutions of the stains made up, and for use to dilute a little with ten times its bulk of distilled water and filter. A solution of good body is thus obtained. Most bacteria (except those of tubercle, leprosy, and a few others) will stain in a short time in such a fluid. Watery solutions may also be made up, *e.g.* a saturated watery solution of methylene-blue or 0·5 per cent. solution of crystal violet. Stains must always be filtered before use ; otherwise there may be deposited on the preparation granules which it is impossible to wash off. The violet stains in solution in water have a great tendency to decompose. Only small quantities should therefore be prepared at a time.

The Staining of Films.—Films are made on slides from *cultures* as described above, and a few drops of the stain are placed on the surface. When the preparation has been exposed for the requisite time it is well washed in tap water or with distilled water. When the film has been washed the surplus of water is drawn off with a piece of filter-paper, the preparation is blotted and carefully dried high over a flame, a drop of cedar-wood oil is placed on the film and the preparation is examined. If the preparation is to be preserved, the cedar-wood oil should be removed by xylol which is then drained off. Such a preparation, if kept free from dust, may be preserved for a long time in the

[1] Bradford, 1924 ; Supplement, 1928.

unmounted state; or it may be mounted in neutral Canada balsam with a cover-glass. In the case of films on cover-glasses, the preparations are dried as above described and then mounted in balsam.

Films of *fluids from the body* (blood, pus, etc.) can be generally stained in the same way, and this is often quite sufficient for diagnostic purposes. The blue dyes are here preferable, as they do not readily over-stain. In the case of such fluids, if the histological elements also claim attention it is best to stain first the cellular protoplasm with 1 to 2 per cent. watery solution of eosin (which is an acid dye), and then to use a blue which will stain the bacteria and the nuclei of the cells. The Romanowsky stains (*vide* p. 129) are here most useful, as by these the preparations are fixed as well as stained.

For the general staining of films a saturated watery solution of methylene-blue or dilute carbol-fuchsin will be found to be the best; the Gram method (*vide infra*) is also essential, and subsequently any special stains which may appear advisable.

Staining of sections may be carried out by the procedures described below; carbol-thionin is specially useful. The following method (Dominici) demonstrates well both organisms and tissue elements: (*a*) Stain for twenty to thirty minutes in a solution of 0·5 gram eosin "water-soluble yellow" and 0·6 gram orange G in 100 c.c. water; (*b*) wash in water (or quickly in 60 per cent. alcohol); (*c*) treat with a watery 0·5 per cent. solution of toluidine blue for a half to two minutes; (*d*) differentiate in 96 per cent. alcohol till stain ceases to come out; (*e*) dehydrate in absolute alcohol, clear in xylol, and mount in balsam. The following method, modified from Stafford, is very simple and satisfactory: (*a*) treat the section for three minutes with a 1 in 5 dilution in water of the following stain—potassium bichromate 1 gram, eosin "water-soluble, yellow," 1 gram, water 100 c.c.; (*b*) wash in water (half a minute) and counter-stain with 1 per cent. aqueous methylene-blue for one minute; (*c*) wash in water, decolorise in methylated spirit, dehydrate in absolute alcohol, clear in xylol and mount in balsam. The modification of Gram's method as applied to sections by Dunlop (p. 119) demonstrates Gram-negative as well as Gram-positive organisms; it is specially recommended.

The Use of Mordants and Decolorising Agents.—In films of blood and pus, and still more so in sections of tissues, if the above methods are used, the tissue elements may be stained to such an extent as quite to obscure the bacteria. Hence many methods have been devised in which the general

principle may be said to be (*a*) the use of substances which, while increasing the staining power, tend to fix the stain in the bacteria, and (*b*) the subsequent treatment by substances which decolorise the overstained tissues to a greater or less extent, while they leave the bacteria coloured. The staining capacity of a solution may be increased :

(*a*) By the addition of substances such as carbolic acid, aniline, or metallic salts.

(*b*) By the addition of alkalis, such as caustic potash or ammonium carbonate, in weak solution.

(*c*) By the employment of heat.

(*d*) By long duration of the staining process.

As decolorising agents are employed chiefly acids (hydrochloric, nitric, sulphuric, acetic), alcohol (either methylated spirit or absolute alcohol), or a combination of spirit and acid, *e.g.* methylated spirit with 1 per cent. of hydrochloric acid added, also aniline and various oils, *e.g.* clove, etc. In most cases about 30 drops of acetic acid in a bowl of water will be sufficient to remove the excess of stain from over-stained films and sections. More of the acid may, of course, be added if necessary. Hot water also decolorises to a certain extent ; over-stained films can often be readily decolorised by placing a drop of water on the film and heating gently over a flame. When preparations have been sufficiently decolorised by an acid, they should be well washed in tap water, or in distilled water with a little lithium carbonate added.

Different organisms take up and retain the stains with various degrees of intensity, and thus duration of staining and decolorising must be modified accordingly.

When methylene - blue, crystal - violet, methyl - violet, or gentian-violet is used, the stain can, after the proper degree of decolorising, be fixed in the tissues by treating for a minute with ammonium molybdate (2½ per cent. in water).

The Formulæ of some of the more commonly used Stain Combinations :

1. *Löffler's Methylene-blue.*

Saturated solution of methylene-blue in alcohol . .	30 c.c
Solution of potassium hydrate in distilled water (1 : 10,000)	100 ,,

(The latter solution may be conveniently made by adding 1 c.c. of a 1 per cent. solution to 99 c.c. of water.)

Sections may be stained in this mixture for from a quarter of an hour to several hours. They do not readily over-stain. The tissue containing the bacteria is then decolorised if necessary with ½–1 per

cent. acetic acid, till it is a pale blue-green. The section is washed in water, rapidly dehydrated with alcohol or aniline-xylol, cleared in xylol, and mounted.

If counter-staining is desired. Dominici's method will be found satisfactory (p. 114).

Films may be stained with Löffler's blue by five minutes' exposure or longer in the cold. They usually do not require decolorisation, as the tissue elements are not over-stained.

2. *Polychrome Methylene-blue.*—This is a methylene-blue solution which has been "ripened" by oxidation so that new violet compounds are formed which stain certain structures, *e.g.* the granules in the *B. diphtheriæ*. Such a solution may be conveniently made from Löffler's methylene-blue by shaking a half-filled bottle from time to time over a long period; several months are necessary to complete the process.

Unna's polychrome methylene-blue solution has the following composition:

Methylene-blue	1 gram.
Carbonate of potassium . . .	1 ,,
Distilled water	100 c.c.

It is "ripened" by the method just described and for use is diluted with 5 to 10 volumes of water.

3. *Carbol-thionin.*

Heat 1 gram thionin in 500 c c. water in the Koch at 100° C. for thirty minutes; cool and add 500 c.c. of 1 per cent. solution in water of carbolic acid; filter. The solution keeps indefinitely.

Stain films or sections for three to five minutes. If more prolonged staining is required, the stain must be washed off with water and a fresh portion filtered on, since on prolonged contact insoluble crystals form on films or sections. Differentiation, if necessary, may be effected by brief treatment with methylated spirit followed by absolute alcohol and xylol.

4. *Rose bengal* 1 per cent. in 5 per cent. watery carbolic acid solution is useful for staining selectively the bacterial bodies where much capsular material is present.

5. *Ziehl-Neelsen Carbol-fuchsin.*—This has the composition

Basic fuchsin (powdered) . .	10 grams.
Alcohol	100 c.c.
Carbolic acid crystals . . .	50 grams.
Water	1000 c.c.

Place the carbolic acid crystals in a flask along with the fuchsin. Mix thoroughly and heat on a boiling water bath for five minutes, shaking occasionally; complete solution occurs. Add the alcohol and mix well. Finally add the water. This is a very powerful stain, and in the undiluted condition is chiefly used for staining acid-fast bacilli. For general purposes it is diluted with fifteen to twenty times its volume of water ("dilute carbol-fuchsin"); stain for ten to fifteen seconds.

Gram's Method and its Modifications.—In the methods already described, the tissues, and more especially the nuclei, retain some stain when decolorisation has reached the point to

which it can safely go without the bacteria themselves being affected. In the method of Gram, now to be detailed, this does not occur, for *the stain can here be removed completely from the ordinary tissues, and left only in the bacteria.* The stain used is a triphenylmethane dye, crystal-violet (or an analogous compound, methyl-violet or gentian-violet). The essential step in the method is the treatment of the preparation with a solution of iodine after staining. The iodine forms with the stain in the bacteria a compound which resists decolorisation with such agents as alcohol, aniline-xylol, acetone, etc., whilst the tissues are decolorised and can then be coloured by a contrast stain. All kinds of bacteria, however, do not retain the stain in this method, and therefore in the systematic description of any species it is customary to state whether or not it retains the violet stain in Gram's method—in other words, whether it is *Gram-positive* or *Gram-negative.* It must also be mentioned that fibrin and some tissue elements may retain the stain as firmly as any bacteria, *e.g.* keratinised epithelium, calcified particles, the granules of mast cells, and sometimes altered red blood corpuscles, etc.

Various modifications of Gram's original method have been introduced, these depending chiefly on the formula of the stain and the decolorising agent used. It is essential that a fresh iodine solution be used. (In some of the modifications iodine is dispensed with.) The following have been selected as suitable :

Weigert's Modification of Gram's Method

The following are the solutions necessary :

(1) Crystal-violet (or Methyl-violet 6 B) . 0·5 gram.
Water . . . . . 100 c.c.

(this is preferable to the original dye solution—carbol-gentian-violet—consisting of a mixture of saturated alcoholic solution of gentian-violet 1 part *plus* 5 per cent. aqueous solution of carbolic acid 10 parts.)

(2) Gram's iodine—
Iodine . . . . 1 gram.
Potassium iodide . . . . . . 2 grams.
Distilled water . . . . . . 300 c.c.

(3) Aniline-xylol—
Aniline . . . . 2 parts.
Xylol . . . . 1 part.

(4) Dilute carbol-fuchsin (p. 116).

A. Films after being fixed are treated as follows :

(1) Stain with violet solution for two to three minutes.

(2) Pour off stain and, without washing, add Gram's iodine; allow to act for about a minute.

(3) Pour off iodine and dry with blotting-paper.

(4) Decolorise with aniline-xylol till the preparation is of a pale violet colour. In the case of films of pus, etc., or sections, decolorising should be controlled under the low power of the microscope till the nuclei have a faint violet colour (the nuclei sometimes retain the violet stain very tenaciously; decolorising may then be aided by breathing slightly on the film while covered with aniline-xylol).

(5) Wash off aniline-xylol thoroughly by means of xylol and then allow to dry.

(6) Counter-stain with dilute carbol-fuchsin for about ten to twenty seconds. Wash in water and dry.

Control Spot.—In order to avoid errors from over-decolorising when examining pus for Gram-negative organisms, it is advisable to make a small film of a known Gram-positive organism (from a twenty-four hours' pure culture, *e.g.* of staphylococcus) at one side of the pus smear; this "control spot" is then treated in the same way as the rest of the film. For the *diagnosis of Gram-negative organisms*, e.g. *gonococcus* or *meningococcus in pus*, it is essential that the "control spot" should retain the Gram's stain vigorously, while the nuclei of the pus cells are at the same time stained pink.

B. Sections should be first stained by carmalum or cochineal-alum [1] for ten minutes. The steps of procedure are then the same as the above, but in stage (5), after being treated with xylol, the preparation is mounted in balsam.

Jensen's Modification of Gram's Method

This is rapidly carried out and gives good results.

Solutions required:

(1) Methyl-violet (6 B)	0·5 gram
Water	100 c.c.
(2) Iodine solution—	
Iodine	1 gram
Potassium iodide	2 grams
Water	100 c.c.
(3) Contrast stain—	
Neutral-red	1 gram.
Acetic acid (1 per cent)	2 c.c.
Water	1000 ,,

Films or sections are treated as follows:

(1) Stain with violet solution for a quarter to a half minute.

(2) Wash off stain with the iodine solution and allow this to act for a half to one minute.

(3) Wash off iodine solution with absolute alcohol and treat with fresh alcohol until the preparation is sufficiently decolorised

(4) Wash off the alcohol with the neutral-red solution and allow the counter-stain to act for about half a minute.

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[1] To 25 grams powdered cochineal ("silver grain") in a flask add 25 c.c. glacial acetic acid; mix thoroughly and add 75 c.c. water. Let the mixture stand at room temperature for twenty-four hours, then add further 400 c.c. water; heat in the Koch for one hour at 100° C.; cool and filter. Mix the filtrate with a solution of 25 grams potash alum in 500 c.c. water and finally add 1 gram salicylic acid. The solution keeps indefinitely.

(5) Wash with water. *Films* are blotted with filter paper and dried. *Sections* are dehydrated with absolute alcohol, cleared in xylol, and mounted.

Kopeloff and Beerman's Modification of Gram's Method

A. FILMS are allowed to dry in air, fixed with the least amount of heat necessary, and treated as follows .

(1) Flood with a freshly prepared mixture of 1 per cent. aqueous crystal-violet solution, 15 parts, with 5 per cent. solution of sodium bicarbonate, 4 parts, and allow to stand for five minutes.

(2) Flush off the excess of stain with iodine solution (2 grams iodine, 10 c.c N/1 NaOH, made up to 100 c.c. with distilled water), then cover with fresh iodine solution and let stand two minutes or longer.

(3) Blot off all free water ; but do not wash or dry.

(4) Decolorise with acetone until the decoloriser flows from the slide practically uncoloured (this usually takes less than ten seconds).

(5) Dry in air

(6) Counter-stain for ten to thirty seconds with dilute carbol-fuchsin—1 : 20

(7) Wash in water, blot, and dry.

In the case of films of pathological material this method should be used for demonstration purposes rather than for determining how an unknown organism reacts to Gram's method, as occasionally coliform bacilli in urinary sediment have appeared to be Gram-positive. This fallacy has not been met with in staining cultures.

B. SECTIONS (according to Dunlop) are treated as above at stages (1) to (4) ; then one proceeds as follows :

(*a*) Wash in water.

(*b*) Counter-stain with Ziehl-Neelsen carbol-fuchsin diluted 1 : 250 with water, for two minutes or longer.

(*c*) Wash in water.

(*d*) Dehydrate quickly with methylated spirit followed by absolute alcohol.

(*e*) Clear in xylol and mount in balsam.

Kirkpatrick's Method for Sections

This method gives very satisfactory results with sections containing Gram-positive organisms, especially where there is difficulty in decolorising the tissues by other methods ; fibrin is not positively stained.

1. Stain with cochineal-alum for ten minutes ; wash in water for a few minutes.
2. Stain with 0·5 per cent. crystal violet for two minutes ; wash in water.
3. Cover the section with 5 per cent. sodium chloride solution for two minutes ; then blot quickly with filter paper (the section must not become " bone dry ").
4. Decolorise with several changes of aniline-xylol till no further

stain is discharged ; wash off the aniline-xylol with xylol. Mount in Canada balsam.

Most bacteria are either frankly Gram-positive or frankly Gram-negative ; but sometimes there is great variability in the avidity with which organisms stained by Gram's method retain the dye when treated with alcohol or other decolorising agent, and it may be difficult to say whether an organism is Gram-positive or not. Such variations may be due to the state of the organisms, the commonest variation being for a Gram-positive organism to become in older cultures Gram-negative. But the result frequently depends on the procedure followed in staining ; the presence of a minute trace of water in the decolorising agent intensifies its action.

Stain for Tubercle and other Acid-fast Bacilli.—These bacilli cannot be well stained with a simple watery solution of a basic aniline dye. This fact can easily be tested by attempting to stain a film of a tubercle culture with such a solution ; with the Gram method, however, a partial staining is effected. Such bacteria require a powerful stain containing a mordant, and must be exposed to the stain for a long time, or its action may be aided by a short application of heat. When once stained, however, they resist decolorising even with very powerful acids ; they are therefore called "acid-fast." The smegma bacillus also resists decolorising with strong acids (p. 429), and a considerable number of other acid-fast bacilli are now known (p. 428). Any combination of crystal-violet or basic fuchsin with aniline or carbolic acid or other mordant will stain the bacilli named, but the following method is most commonly used[1] :

1. Filter enough Ziehl-Neelsen carbol-fuchsin (p. 116) on to the film or section to cover it well, heat till steam rises, and repeat this several times, allowing the stain to act for five minutes—the surface must not be allowed to dry ; if necessary add further stain during the process ; or place the preparation in the cold stain for twelve to twenty-four hours. (Films and paraffin sections are usually stained with hot stain, loose sections with cold ; in hot stain the latter shrink.)
2. Decolorise with 20 per cent. solution of strong sulphuric acid, nitric acid, or hydrochloric acid, in water. In this the tissues become yellow.
3. Wash well with water. The tissues will regain a faint pink tint. If the colour is distinctly red, the decolorisation is insufficient, and the specimen must be returned to the acid. As a matter of

[1] A staining solution containing crystal-violet instead of basic fuchsin may be used by those who have difficulty in discriminating between red and blue tints. In this case chrysoidin (1 : 300 in water) is used as the counter-stain.

practice, it is best to remove the preparation from the acid every few seconds and wash in water, replacing the specimen in the acid and re-washing till the proper pale pink tint is obtained. Then wash in alcohol for half a minute, and replace in water.

4. Contrast stain with a saturated watery solution of methylene-blue for half a minute.

5. Wash well with water. In the case of *films*, blot and dry. In the case of *sections*, dehydrate, clear, and mount.

In specimens stained by the Ziehl-Neelsen method acid-fast bacilli are bright red. Other bacteria which may be present, and cells or tissues, are coloured blue.

Tubercle bacilli, which are intensely acid-fast, retain the stain even after treatment with the acid for many hours; but other acid-fast bacilli may become decolorised in a few minutes.

Leprosy bacilli are stained in the same way, but are often rather more easily decolorised than tubercle bacilli, and it is better to use only 5 per cent. sulphuric acid in decolorising.

Stains for Diphtheria Bacillus.—The following methods for bringing out the structural characters of this organism may be recommended. In the case of cultures, films should be made from eighteen to twenty-four hours' growth on serum medium.

Neisser's Stain (second or modified method)

1. Stain films for a few seconds in a mixture of solutions A and B, two parts of the former to one of the latter.

A.	Methylene-blue . . .	1 gram.
	Absolute alcohol . . .	50 c.c.
	Glacial acetic acid . . .	50 ,,
	Distilled water . . .	1000 ,,
B.	Crystal-violet . . .	1 gram.
	Absolute alcohol . . .	10 c.c.
	Distilled water . . .	300 ,,

2. Wash for a few seconds in soft or distilled water (this stage may be omitted with advantage).

3. Stain in chrysoidin-solution (1 : 300) for a few seconds (the chrysoidin should be dissolved in warm water and the solution then filtered).

4. Wash quickly in water, blot, and dry.

The substance of the bacilli is brownish yellow, the granules are almost black.

Instead of chrysoidin the following solution of erythrosin may be used with advantage: Saturated alcoholic solution of erythrosin, 20 parts; saturated watery solution of picric acid, 90 parts; add to the mixture precipitated calcium carbonate to excess; allow to stand for a time, shaking at intervals; filter.

Pugh's Stain

The staining solution consists of

Toluidine-blue	0·1 gram.
Absolute alcohol	2 c.c.
5 per cent. solution of glacial acetic acid in distilled water	100 ,,

Stain for two to three minutes. Wash with water and dry. The substance of the diphtheria bacilli is light blue and the granules are of a reddish purple tint.

The Staining of Spores.—If bacilli containing spores are stained with a watery solution of a basic aniline dye the spores remain unstained. The spores either take up the stain less readily than the protoplasm of the bacilli, or they have a resisting envelope which prevents the stain from penetrating to the protoplasm. Like tubercle bacilli, when once stained they retain the colour with considerable tenacity. In fixing the films little heat should be used, as overheating interferes with the subsequent staining. The following is the simplest method for staining spores:

1. Stain films with carbol-fuchsin (p. 116), heating as for tubercle bacilli.
2. Wash in water and treat with 30 per cent. solution of ferric chloride for one to two minutes.
3. Without washing treat with 5 per cent. sodium sulphite solution for fifteen to thirty seconds (according to Armstrong).
4. Wash in water.
5. Stain with 1 per cent. aqueous methylene-blue for half to one minute
6. Wash in water, and dry.

The result is that the spores are stained red, the protoplasm of the bacilli blue.

The spores of some organisms lose the stain more readily than those of others.

The Staining of Capsules.—The following methods may be recommended in the case of capsulated bacteria:

1. *Hiss's Method*

In this method the staining solution consists of 1 part of a saturated alcoholic solution of fuchsin or gentian-violet and 19 parts of distilled water. A few drops of the stain are placed on a film, previously dried and fixed by heat, and the preparation is steamed for a few seconds over a flame. The staining solution is washed off with a 20 per cent. solution of copper sulphate; the preparation

(without being washed in water) is dried between filter papers. The capsules of pneumococci in exudates or growing in a fluid serum medium can be readily demonstrated by this method; in the case of solid cultures, films should be made without any diluent, or a drop of fluid serum should be used. The method is easily applied, and gives excellent results.

2. *Richard Muir's Method* (modified)[1]

1. The film containing the bacteria must be very thin. It is dried and stained in filtered carbol-fuchsin for half a minute, the preparation being gently heated.
2. Wash slightly with spirit and then well in water.
3. Place in following mordant for a few seconds:

Saturated solution of corrosive sublimate	2 parts.
Tannic acid solution—20 per cent.	2 ,,
Saturated solution of potash alum	5 ,,

4. Wash well in water.
5. Treat with methylated spirit for about a minute.

The preparation has a pale reddish appearance.

6. Wash well in water.
7. Counter-stain with ordinary watery solution of methylene-blue for half a minute.
8. Dehydrate in alcohol, clear in xylol, and mount in balsam.

The bacteria are a deep crimson, and the capsules of a blue tint. The capsules of bacteria in certain culture media may be demonstrated by this method.

3. *J. Kirkpatrick's Method*

Solutions required:

(1) Fixing solution—

Formalin	10 c.c.
Chloroform	30 ,,
Absolute alcohol	60 ,,

(2) Manson's borax methylene-blue—

Methylene-blue	1 gram.
Borax	2 grams.
Water	100 c.c.

Dissolve borax in boiling water over flame, add methylene-blue, cool, and filter. For use take

Borax methylene-blue	1 part.
Distilled water	5 parts.

(1) Make film on slide, then allow to dry in air (the film must not be heated).

(2) Pour on fixing solution and allow to remain for one to three minutes.

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[1] For a method of staining capsules of bacteria in sections, reference may be made to Rd. Muir, *Journ. Path. and Bact.*, xx. (1916), 257.

(3) Wash off with spirit; drain off excess and stain with dilute borax methylene-blue for one to three minutes.

(4) Wash quickly in water, blot, and dry; or dehydrate in acetone, clear in xylol, and mount in balsam.

4. *Relief Staining*

Capsules can also be demonstrated by the India-ink method (p. 128), or by the following procedure (Howie and Kirkpatrick) which is very satisfactory.

Staining solution:

(1) 10 per cent water-soluble eosin ("yellowish" or "bluish") or erythrosin in distilled water .	4 parts
(2) Serum (human, rabbit, sheep or ox, heated at 56° C.)	1 part
(3) Crystal of thymol.	

The mixture is allowed to stand at room temperature for several days and is then centrifuged and the supernatant fluid stored at room temperature; it keeps for many months.

On a clean slide with a platinum loop of about 1 mm. diameter, one drop of exudate (or fluid culture or a suspension in *broth* from an agar slope culture) is mixed with one drop of Ziehl-Neelsen's carbol-fuchsin solution diluted 1 : 5 and allowed to stain for half a minute. Then one drop of eosin solution is added and the slide left for about 1 minute. After this a film is spread with cigarette paper in the manner of a blood film. It dries rapidly and may be examined in cedar-wood oil, or may be fixed by gentle heating, but such fixation tends to increase the granularity of the background and should be avoided. The appearances do not alter when the films are kept for many months. (In the case of cocci the treatment with Ziehl-Neelsen's solution can usually be omitted.)

The Staining of Flagella.—The staining of the flagella of bacteria requires considerable practice to ensure that good results shall be obtained. Many methods have been introduced, of which the two following are very satisfactory:

1. *J. Kirkpatrick's Method*

Solutions required:

Fixing Solution

Absolute alcohol . . .	60 c.c.
Chloroform	30 c.c.
Formalin	10 c.c.

Mordant

Ferric chloride, 5 per cent. solution . .	1 part
Tannic acid, 20 per cent. solution (dissolved by heat and allowed to cool) . . .	3 parts

Before use dilute mordant with an equal volume of water.

Silver Stock Solution

Place 10 grams of silver sulphate (B.D.H.) in a clean *clear glass* bottle and add 200 c.c. distilled water. Incubate at 37° C. for twenty-four hours, shaking occasionally. The solution improves by leaving it exposed to daylight and keeps indefinitely.

Silver Staining Solution.—Rinse a clean 100 c.c. Erlenmeyer flask with distilled water. Place 40 c.c. of filtered silver stock solution in the flask and add quickly 0·6 c.c. ethylamine " 33 per cent. W.V." (B.D.H.). A precipitate forms which is immediately redissolved. From a clean pipette (made from a piece of unused quill tubing) add filtered silver stock solution until a permanent, slightly opalescent solution results. Finally, add 10 c.c. of distilled water.

Bacterial Suspension.—In the case of the coli-typhoid group agar cultures which have been grown at 37° C. for forty-eight hours are best; for other organisms, *e g.* vibrios, twenty-four-hours' cultures may be preferable. Heat a clean dry 3-inch by ½-inch test-tube in the Bunsen flame and allow to cool. Place about 1 c.c. sterile distilled water in the tube. With a platinum wire remove a loopful of the agar culture, taking care not to break the surface of the medium. Suspend the loopful of the culture in the distilled water and gently rotate the loop until an even suspension is obtained. Add distilled water to give a suspension of about 200 million organisms per c.c.

Cleaning of Slides.—The slides used for making films should be cleaned in the bichromate-nitric mixture (p. 109), thoroughly washed, and dried with a clean cloth, and finally passed through the Bunsen flame to remove any traces of grease. They are allowed to cool before spreading the films.

Procedure :

(1) On a clean slide place one loopful of bacterial suspension. Spread the film with a platinum wire, drawing excess of fluid to one end of the film : this thicker part of the film acts as a control spot in the staining process. Allow to dry in the air.

(2) Place film in fixing solution in jar for one to three minutes.

(3) Rinse in spirit, then wash thoroughly in water.

(4) Treat with mordant for three to five minutes in jar.

(5) Wash well in water ; dry the under side of the slide.

(6) Lay the slide on a staining rack over the sink ; filter on silver staining solution. Heat the slide by flaming the under side with a throat swab dipped in methylated spirit. Continue heating, keeping the flame moving, until the control spot appears of a dark brown colour and a metallic scum forms on the edges of the fluid—about fifteen seconds. Cease flaming and allow the heated silver solution to act for fifteen to thirty seconds longer.

(7) Wash off the staining solution in running water (the staining solution must not be poured off or deposit will form on the film).

(8) Dry the film and mount in balsam.

A well-stained film shows the organisms uniformly black, the flagella being clearly defined and of a light brown-black or grey colour. Flagella showing a granular appearance may be due to the staining solution containing excess of ethylamine, by over-

heating while staining, or by over-treatment in mordant. A crystalline deposit in the preparations is caused either by excessive heating while staining, or insufficient ethylamine in the staining solution. A silver staining solution which contains excess of ethylamine may be corrected by the addition of a few drops of the stock silver solution ; one which contains too little ethylamine had better be rejected.

2. *Pitfield's Method as modified by Richard Muir*

Prepare the following solutions :

A. *The Mordant.*

Tannic acid, 10 per cent. watery solution, filtered .	10 c.c.
Corrosive sublimate, saturated watery solution .	5 ,,
Alum, saturated watery solution	5 ,,
Carbol-fuchsin (*vide* p. 116)	5 ,,

Mix thoroughly. A precipitate forms, which must be allowed to deposit, either by centrifugalising or simply by allowing to stand. Remove the clear fluid with a pipette, and transfer to a clean bottle. The mordant keeps well for one or two weeks.

B. *The Stain.*

Alum, saturated watery solution . . .	10 c.c.
Gentian-violet, saturated alcoholic solution .	2 ,,

The stain should not be more than two or three days old when used. It may be substituted in the mordant in place of the carbol-fuchsin.

A bacterial suspension having been prepared as above described, a film is made by spreading a drop on a clean cover-slip and is allowed to dry in the air ; it is then passed twice or thrice through a flame, care being taken not to overheat it. Pour over it as much of the mordant as the cover-glass will hold. Heat gently over a flame till steam begins to rise, allow to steam for about a minute, and then wash well in a stream of running water for about two minutes. Then dry carefully over the flame, and when thoroughly dry pour on some of the stain. Heat as before, allowing to steam for about a minute, wash well in water, dry, and mount in a drop of xylol balsam.

Staining of Spirochætes in Sections.—The following impregnation methods have been applied for this purpose and give excellent results :

1. *Levaditi's Original Method*

(1) The tissues, which ought to be in thin slices, about 1 mm. in thickness, are best fixed in 10 per cent. formalin solution for twenty-four hours.

(2) They are washed for an hour in water, and then brought into 96 per cent. alcohol for twenty-four hours.

(3) They are then placed in 1·5 per cent. solution of nitrate of

silver in a dark bottle, and are kept in an incubator at 37° C. for three days.

(4) They are washed in water for about twenty minutes, and are thereafter placed in the following mixture, namely :

Pyrogallic acid	4 grams.
Formalin	5 c.c.
Distilled water up to . . .	100 ,,

They are kept in this mixture in a dark bottle for forty-eight hours at room temperature.

(5) They are then washed in water for a few minutes, taken through increasing strengths of alcohol, and embedded in paraffin in the usual way. The sections, which ought to be as thin as possible, are fixed on slides as usual ; the paraffin is removed by xylol and the preparation is mounted in balsam. In satisfactory preparations the spirochætes appear of an almost black colour against the pale yellow background of the tissues. The latter can be counter-stained by weak carbol-fuchsin or by toluidine-blue.

2. *Jahnel's Method for Nervous Tissue, etc.*

(1) Wash in water for one to three days thin pieces (2–4 mm.) of tissue which have been fixed in 10 per cent. formalin for at least fourteen days.

(2) Place in pure pyridine for one to three days.

(3) Wash in many changes of water until the smell of pyridine is practically gone—two to three days.

(4) Place the pieces in 5 to 10 per cent formalin for a few days.

(5) Wash thoroughly in water.

(6) Place for half to one hour in fresh 1 per cent. solution of uranium nitrate (Merck) in distilled water at 37° C. Penetration is assisted by letting the tissue rest on a layer of lead-free glass wool. (The uranium nitrate prevents subsequent staining of other elements in nervous tissue.)

(7) Wash for one day in distilled water.

(8) Place in 96 per cent. alcohol for three to eight days.

(9) Wash in distilled water till the tissue sinks.

(10) Place in 1·5 per cent. solution of silver nitrate crystals and leave five to eight days at 37° C. The container should be dark.

(11) Decant off the silver nitrate solution, wash the tissue slightly in water in the dark, then transfer it for twenty-four to forty-eight hours at room temperature in the dark to a mixture made up as follows :

4 per cent. watery pyrogallic acid	90 c.c.	reject 15 c.c. of this solution
acetone	10 c.c.	and replace by pyridine.

(12) Wash in distilled water, dehydrate in neutral alcohol, clear and embed in paraffin.

It should be noted that spirochætes stained with silver rapidly become colourless on contact with certain immersion oils.

Examination of Spirochætes in Films.—The following methods may be recommended :

1. *Fontana's Method*

This is a silver impregnation method; three solutions are necessary as follows:

(*a*) *Fixative.*—Acetic acid 1 c.c., formalin 2 c.c., and water 100 c c.
(*b*) *Mordant.*—5 per cent. tannic acid in a 1 per cent. watery solution of carbolic acid.
(*c*) *Stain.*—$\frac{1}{4}$ per cent. solution of silver nitrate in distilled water. For use a small quantity of this is put in a test-tube, and a minute amount of ammonia solution is added till there is distinct turbidity. (If too much ammonia is added the fluid becomes clear again.)

Dried films, which should not be fixed by heat, are fixed in solution (*a*) for about a minute, the fluid being dropped on the film and renewed once or twice. The preparation is then washed thoroughly in running water, solution (*b*) is dropped on the film, heated till steam rises, and allowed to remain for about half a minute. It is again washed in water, solution (*c*) is dropped on, heated till steam rises, and allowed to remain for another half minute. The preparation is finally washed in water and dried.

The spirochætes are of a dark brown or black colour, and are easily found. This is an excellent method, and is easily carried out.

2. *Becker's Method* (modified)

The *Fixative* and *Mordant* used are the same as in Fontana's method (see above).

Staining Solution

Basic fuchsin (saturated alcoholic solution)		45 c.c.
Shunk's mordant B (absolute alcohol or 95 per		
cent ethyl alcohol	16 c.c.	18 c.c.
aniline	4 c.c.	
Distilled water		100 c.c.

Mix the Shunk's mordant with the alcoholic fuchsin solution, then add the distilled water. The glassware should be thoroughly dry, or rinsed out with alcohol before use.

The stain and reagents are filtered into jars for use.

Procedure:

(1) Make film on slide and allow to dry.
(2) Place in fixative for one and a half to three minutes.
(3) Wash in water (half a minute) and treat with mordant for three to five minutes.
(4) Wash in water (half a minute) and place in staining solution for three to five minutes.
(5) Wash in water, dry, and mount in balsam.

3. *India-ink and Collargol Method*

In the first, an emulsion of India-ink of fine quality is sterilised by steaming and allowed to settle for a few days; a drop of the deposit diluted with an equal quantity of distilled water is well

rubbed up and spread on a slide with a drop of the material to be examined (exudate from chancre or condyloma, scraping from congenitally affected organ, etc.). The film is dried and examined with an immersion lens without the interposition of a cover. Spirochætes, if present, stand out unstained, surrounded by the dark India-ink, and often positive results are rapidly obtained by means of it. The organisms are not so readily recognised by this method as by dark-ground illumination, and negative observations are thus less valuable.

Harrison, in place of India-ink, uses a suspension of collargol in distilled water (1 : 19), which should be shaken before use. It is employed in a similar way.

4. *Giemsa's Stain* (p. 131)

The Romanowsky Stain.—This stain and its modifications are extensively used for demonstrating protozoa (malaria parasites, trypanosomes, etc.), spirochætes, Rickettsia bodies and viruses, and the blood cells, as well as tissues and bacteria. The dye concerned is the compound which is formed when watery solutions of medicinal methylene-blue and water-soluble eosin are brought together. This compound is insoluble in water but soluble in methyl alcohol. The stain was originally used by Romanowsky for the malarial parasite, and its special quality is that it imparts to certain elements, such as the chromatin of this organism, a reddish-purple hue. This was at first thought to be simply due to the combination of the methylene-blue and the eosin, but it is now recognised that certain changes, such as occur in methylene-blue solutions with age, are necessary. In the modern formulæ these changes are brought about by treatment with alkalis, especially alkaline carbonates, as was first practised by Unna in the preparation of his polychrome methylene-blue. The stains in use thus contain a mixture of methylene-blue and its derivatives in combination with eosin; the differences in these bodies and the different proportions in which they occur in individual stains, as also differences in the reaction of the staining fluid, account for the different effects produced on the various constituents of a cell. The underlying chemical reactions are complicated and as yet not fully understood. Thus it is not certainly known to what particular new body the reddish hue produced in chromatin is due, but the active constituent may be methylene-violet or methylene-azure, or thionin, all of which result from the action of alkali on methylene-blue.

The following are the chief formulæ in use:

1. *Jenner's Stain.*—This is an excellent blood stain, but is not so good for the study of parasites as the others to be mentioned.

The stain is supplied as a powder, and of this 0·5 gram is dissolved in 100 c.c. pure methyl alcohol. For use a few drops are placed on the dried unfixed film for one to three minutes, the dye is poured off, and the preparation washed with distilled water till it presents a pink colour ; it is then dried between filter-paper and mounted in balsam.

2. *Leishman's Stain.*—This is supplied as a powder or as a solution already made up. Of the powder, 0·15 per cent. is added to pure methyl alcohol [1] (" for analysis, acetone free "). The mixture is shaken occasionally during the course of two to three days and, after being allowed to settle, is ready for use. The stain will keep for a long period

Method.—Blood films are allowed to dry before being stained.

(1) Support the slide or cover-glass in a horizontal position and raised off the bench.

(2) Drop on the stain from a pipette until the whole surface is uniformly covered (5–10 drops for a cover-glass ; 20–30 drops for a slide). Allow to act for one and a half minutes.

(3) With the same pipette, add 1 or 2 drops of distilled water (or soft tap water) for each drop of stain used under (2). The fluids will mix thoroughly without any rocking of the slide or cover-glass, and at least one quarter of an hour, and preferably a half to one hour or longer, is allowed for staining to be effected

(4) Do not pour off the stain, but allow a gentle stream of water (*soft* tap water, or distilled water) to wash it away. The film should appear bright pink in colour, and the washing may be continued for a half to two minutes.

(5) Drain off excess of water and allow the stained film to dry in air (do not heat).

(6) Mount in neutral Canada balsam.

The results are most satisfactory with freshly prepared films or with such as are only a few days old. If older than this, there is an increasing tendency for the red cells to stain of a dusky bluish colour or else for them to remain practically unstained while the surrounding dried plasma exhibits markedly basophile characters. It is to be noted, however, that malarial parasites, spirochætes, etc., remain readily recognisable in spite of such altered staining affinities on the part of the red blood corpuscles.

If the staining is too blue, differentiation may be obtained by washing with 1–0·1 per cent. boric acid or monosodium phosphate solution and then with water ; the phosphate is the more intense in its action.

For staining sections a little modification is necessary. A paraffin section is taken into distilled water as usual, the excess of water is drained off, and a mixture of one part of stain and two parts of distilled water is placed on it. The stain is allowed to act for five to ten minutes till the tissue appears a deep Oxford blue ; it is then decolorised with 1 : 1500 acetic acid—the effect being watched under a low-power lens. The blue begins to come out, and the process is allowed to go on till only the nuclei remain blue. The section

[1] Merck's methyl alcohol ("for analysis, acetone free") is usually specified. "Purified" methyl alcohol is fairly satisfactory provided that the pH is adjusted to 6 5 ; if the alcohol is too acid the reaction, as tested by the usual indicators, may be corrected by the addition of N/100 NaOH.

is then washed with distilled water, rapidly dehydrated with alcohol, cleared, and mounted. If, as sometimes happens, the eosin tint be too well marked, it can be lightened by the action of 1 : 7000 solution of caustic soda, this being washed off whenever the desired colour has been attained.

In certain cases, *e.g.* for the staining of old films or of trypanosomes or Leishmaniæ in sections, Leishman recommends an initial treatment of the preparation with serum. This modification is described below (p. 824).

3. *J. H. Wright's Stain.*—This stain, which is much used in America, is applied in almost the same way as Leishman's. A few drops of the solution in methyl alcohol are placed on the preparation for a minute for fixation ; water is then dropped on till a green iridescent scum appears on the top of the fluid, and staining goes on for about two minutes ; the stain is then washed off with distilled water, and a little is allowed to remain on the film till differentiation is complete ; the preparation is carefully dried with filter paper.

4. *Giemsa's Stain.*—According to Giemsa the reddish-blue hue characteristic of the Romanowsky stain is due to the formation of methylene-azure, and he has prepared this by a method of his own under the name " Azur I." From this, by the addition of an equal part of medicinal methylene-blue, he prepares what he calls " Azur II.," and from this again by the addition of eosin he prepares "Azur II.-eosin." The latest formula for the finished stain is as follows : Azur II.-eosin, 3 grams ; Azur II., 0·8 gram ; glycerol (Merck, chemically pure), 250 grams ; methyl alcohol (Kahlbaum, I.), 250 grams. This stain has been extensively used for demonstrating spirochætes, but it can be used for any other purpose to which the Romanowsky stains are applicable. For spirochætes the following are Giemsa's directions :

(1) Fix films in absolute alcohol for fifteen to thirty minutes, dry with filter-paper. (2) Dilute stain with distilled water[1] immediately before use—one drop of stain to 1 c.c. water (the mixture being gently shaken). (Sometimes the water is made alkaline by the addition of one drop of 1 per cent. potassium carbonate to 20 c.c water.) (3) Stain for thirty minutes ; for spirochætes a longer period is desirable (even twenty-four hours), the stain being renewed. When prolonged staining is required the slide or cover-slip should be immersed in the fluid with the film side down, to avoid deposit. (4) Wash in brisk stream of distilled water. (5) Drain with filter-paper, dry, and mount in Canada balsam.

The following is a rapid method suitable for spirochætes. The film, fixed as above or by drawing three times through the flame, is covered with the diluted stain and warmed till steam rises ; after fifteen seconds this is poured off and fresh stain added, the film is again heated ; this procedure is repeated four or five times. Finally, the film is washed in water and dried.

Old films containing protozoa tend not to differentiate properly. To remedy this, after staining and brief, brisk rinsing in water, place them film-side up in a Petri dish containing a 1 : 1000 solution of NaH_2PO_4 in water to each 10 c.c. of which 2–3 drops of 1 : 1000 solution of eosin have been added. Differentiation occurs in about

[1] The distilled water must not be acid. To drive off CO_2 it shou'd be boiled for five to ten minutes in a flask ; it should be kept stoppered.

one to four minutes and is indicated by a delicate reddish brown tint on naked eye examination. Another method is to treat the film with formol-alcohol (p. 111), rinse in water and then use the diluted stain.

Sabouraud's Method for Staining Trichophyta.—Remove the fat from the hair or epithelial squames with chloroform. Place in a test-tube with 10 per cent. formalin, and warm for two or three minutes till ebullition commences. Wash well in distilled water, and stain for one minute in Sahli's blue, which is made up as follows:

Distilled water	40 parts.
Saturated watery methylene-blue .	24 ,,
5 per cent. solution of borax in water	16 ,,

Mix the constituents. Allow to stand for a day, and filter.

After staining, wash in water, dehydrate with absolute alcohol, clear in xylol, and mount in balsam.

The Fixation and Hardening of Tissues.—The following are among the best methods for bacteriological purposes:

(*a*) *Formalin Solution.*—This may be used as a 10 per cent. dilution of the commercial solution (40 per cent. formaldehyde) with water. Thin pieces of tissue are fixed in this for twenty-four hours; they are then placed in 50 per cent. spirit for twenty-four hours, and then in pure spirit.

When blocks of tissue are kept in formalin for a long time deposits tend to form. These may be removed by soaking the blocks over night in a 1–2 per cent. dilution of strong ammonia in water and then washing for several hours prior to embedding.

(*b*) *Corrosive sublimate* is an excellent fixing agent; it is best used as a saturated solution in water. Dissolve 70 grams sublimate in 1000 c.c. water by heat; the separation of crystals on cooling shows that the solution is saturated. For small pieces of tissue $\frac{1}{8}$ inch in thickness, four to twelve hours' immersion is sufficient; if the pieces are larger, twenty-four hours is necessary. Fixation is complete when the tissues have acquired a uniform whitish colour throughout. They should then be placed in a stream of running water for from twelve to twenty-four hours, according to the size of the pieces, to wash out the excess of sublimate. They are then placed for twenty-four hours in each of the following strengths of methylated spirit (free from naphtha[1]): 30 per cent., 60 per cent., and 90 per cent. Finally, they are placed in absolute alcohol[2] for twenty-four hours, and are then ready to be prepared for cutting. Washing in water may be omitted, the tissue being transferred directly from sublimate to 60 per cent. alcohol.

If the tissue is very small, as in the case of minute pieces removed

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[1] In Britain ordinary commercial methylated spirit has mineral naphtha added to it to discourage its being used as a beverage. The naphtha being insoluble in water a milky fluid results from the dilution of the spirit. By law, chemists can only sell 8 ounces of pure spirit at a time. Most pathological laboratories are, however, permitted by the Excise to buy "industrial spirit," which contains only one-nineteenth of wood naphtha.

[2] "Absolute industrial spirit" may be used instead of absolute alcohol in the dehydrating and embedding processes.

for diagnosis, the stages may be all compressed into twenty-four hours.

In tissues which have been fixed with sublimate, deposits are frequent. These may be removed from sections, at the stage before staining, by treatment for about five minutes with liquor iodi mit. made up with methylated spirit, the result being controlled under the microscope. The iodine is removed by treatment with alcohol followed by 0·25 per cent. sodium thiosulphate solution and then washing in water.

(*c*) *Helly's Fluid* (Zenker's solution with formalin instead of acetic acid).—Potassium bichromate, 2·5 grams; sodium sulphate, 1 gram; corrosive sublimate, 5 grams; water, 100 c.c. Before use add 5 c.c. of formalin to 100 c.c. of fixing fluid. Pieces of tissue should be small and fixed for not more than eighteen to twenty-four hours. They are then washed in running water for twenty-four hours and treated with successive strengths of alcohol as after fixation with corrosive sublimate.

The Cutting of Sections.—A. *By Means of the Freezing Microtome.*—Pieces of tissue hardened by any of the above methods must have all the alcohol removed from them by washing in running water for twelve to twenty-four hours. They are then placed for twelve to twenty-four hours (according to their size) in a thick syrupy solution containing 2 parts of mucilage acacia and 1 part of syrup. They are cut on a freezing microtome and placed for a few hours in a bowl of water so that the gum and syrup may dissolve out. They are then stained, or they may be stored in methylated spirit.

B. *Embedding and Cutting in Solid Paraffin.*—This method gives by far the finest results, and should always be adopted when practicable. The principle is the impregnation of the tissue with paraffin in the melted state. This paraffin when it solidifies gives support to all the tissue elements. The method involves that, after hardening, the tissue shall be thoroughly dehydrated, and then thoroughly permeated by some solvent of paraffin which will expel the dehydrating fluid and prepare for the entrance of the paraffin. Various solvents are in use; of these, chloroform is the most suitable. The more gradually the tissues are changed from reagent to reagent the more successful is the result. In the final stage the tissues are kept in paraffin just above its melting-point in an oven the temperature of which is controlled by a thermostat. The tissues tend to become brittle if over-heated, and therefore the best results are obtained by using paraffin melting at a temperature between 52° and 53° C. The successive steps in the process of paraffin embedding are as follows:

1. Pieces of tissue, after having been hardened and brought through spirit, are placed in fresh absolute alcohol for twenty-four hours in order to effect complete dehydration.
2. Transfer now to a mixture of equal parts of absolute alcohol and chloroform for twenty-four hours.
3. Transfer to pure chloroform for twenty-four hours or longer. At the end of this time the tissues should sink or float heavily.
4. Transfer now to a mixture of equal parts of chloroform and paraffin, and place on the paraffin oven for from twelve to twenty-four hours.
5. Place in pure melted paraffin in the oven for twenty-four hours. For holding the paraffin containing the tissues, small tin dishes such

as are used by pastry-cooks will be found very suitable. There must be a considerable excess of paraffin over the bulk of tissue present, otherwise sufficient chloroform will be present to vitiate the final result and not give the perfectly hard block obtained with pure paraffin. With experience, the persistence of the slightest trace of chloroform can be recognised by smell.

In the case of very small pieces of tissue the time given for each stage may be much shortened, and where haste is desirable Nos. 2 and 4 may be omitted. Otherwise it is better to carry out the process as described.

6. Cast the tissues in blocks of paraffin as follows: Pairs of L-shaped pieces of metal made for the purpose by instrument makers must be at hand. By laying two of these together on a glass plate, a rectangular trough is formed. This is filled with melted paraffin taken from a stock in a separate dish. In it is immersed the piece of tissue, which is lifted out of its pure paraffin bath with heated forceps. The direction in which it is to be cut must be noted before the paraffin becomes opaque. When the paraffin has begun to set, the glass plate and trough have tepid water run over them When the block is cold, the metal L's are broken off, and, its edges having been pared, it is stored, *e.g.* in a pill-box.

For tissues which have a special tendency to become hard, e.g. bowel, blood vessels, dehydration with butyl alcohol is useful. The procedure is as follows (modified from Stiles): pieces of tissue from water or 50 per cent. spirit are placed for three to six hours successively in mixtures of:

Butyl Alcohol (technical)	*Ethyl Alcohol.*
10 parts . . .	45 per cent.—90 parts
25 ,, . . .	70 ,, —75 ,,
55 ,, . .	90 ,, —45 ,,
75 ,, . . .	100 ,, —25 ,,

and then for twelve to twenty-four hours in each of three successive changes of butyl alcohol. Thereafter the tissue is placed for four to eight hours in a mixture of equal parts of butyl alcohol and paraffin in the oven and finally in pure paraffin for four to eight days, the paraffin being changed once during this time.

The Cutting of Paraffin Sections.—Sections must be cut as thin as possible, the Cambridge rocking microtome being, on the whole, most suitable. They should not exceed 8 μ in thickness and ought, if possible, to be about 4 μ. When cut, sections are floated on the surface of a beaker of water kept at a temperature about 10° C. below the melting-point of the paraffin. On the surface of the warm water they become perfectly flat. They are then fixed on slides.

Fixation on Ordinary Slides.—(*a*) *Gulland's Method.*—A supply of slides well cleaned being at hand, one of them is thrust obliquely into the water below the section, a corner of the section is fixed on it with a needle, and the slide withdrawn. The surplus of water being wiped off with a cloth, the slide is placed on a support, with the section downwards, and allowed to remain on the top of the paraffin oven or in a bacteriological incubator for from twelve to twenty-four hours. It will then be sufficiently fixed on the slide to withstand all the manipulation necessary during staining and mounting.

Sections of tissues fixed in Helly's fluid or other bichromate solution cannot be fixed to slides by this method ; albuminised slides, as in Mann's method, should be used.

(*b*) *Fixation by Mann's Method.*—This has the advantage of being more rapid than the previous one. A solution of albumin is prepared by mixing the white of a fresh egg with 10 parts of distilled water and filtering ; this is preserved with thymol. Slides are made perfectly clean with alcohol. One is dipped into the solution and its edge is then drawn over one surface of another slide so as to leave on it a thin film of albumin. This is repeated with the others As each is thus coated it is leant, with the film downwards, on a ledge till dry, and then the slides are stored in a wide stoppered jar till needed. The floating out is performed as before. The albuminised side of the slide is easily recognised by the fact that if it is breathed on, moisture does not condense on it. The great advantage of this method is that the section is fixed after twenty to thirty minutes' drying at 37° C.

CHAPTER IV

SEROLOGICAL METHODS—PREPARATION OF VACCINES—INOCULATION OF ANIMALS—METHODS OF OBTAINING PATHOLOGICAL MATERIAL FOR EXAMINATION

The Testing of Agglutinating Properties of Serum

In studying the properties of serum it is necessary to have the means of measuring and diluting small quantities of fluid. The simplest method is by means of 1 c.c. and 0·1 c.c. pipettes. Each pipette should be graduated in tenths, and should deliver to the end.

Method of using Pipettes.—A mouthpiece of glass quill tube is attached to the pipette by indiarubber tubing. To obviate the danger of sucking up living cultures into the mouth a small piece of cotton wool may be packed loosely into one end of the indiarubber tube. The mouthpiece is held in one corner of the mouth between the teeth, and the junction between the glass stem of the pipette and the attached indiarubber tube is held between the thumb and first and second fingers of the right hand. In order to draw up a measured amount, say 0·05 c.c., into the fine pipette—(1) draw up fluid to slightly above the 0·05 mark, then apply the tip of the tongue to the orifice of the mouthpiece, so as to close it, and continue to keep the tongue in this position until operation (4) is completed; (2) withdraw the nozzle of the pipette from the fluid, and bring it against the side of the vessel above the level of the fluid; (3) while maintaining the nozzle continuously against the vessel wall make slight pressure on the rubber with the fingers and thumb of the right hand until the level of the fluid in the pipette reaches the 0·05 mark; then do not make any further pressure; (4) then insert the pipette into the vessel into which the fluid is to be delivered (if the amount to be delivered is very small, *e.g.* 0·01 c.c., pass the nozzle down to within a short distance of the fluid in the tube) and (5) blow out vigorously—while blowing, bring the nozzle into contact with the side of the tube above the level of the contents. (The object of bringing the nozzle against the vessel wall in (3) and (5) is to ensure that the fluid shall drain away from the pipette and not remain at the nozzle as a drop, which would cause a serious error in the measurement of the amount delivered.) Fig. 24 shows the mode of holding the pipette, the reader being in the same position as the operator. (The pipette must be well washed out with saline between

successive reagents. Traces of very active substances such as toxins or antisera may be destroyed by boiling, but care must be taken that albuminous matter does not coagulate in the pipette.)

The Drop Method.—Another method of measuring fluids depends on the use of dropping pipettes. When one pipette is used throughout, the procedure is simple, and it is accurate when fluids such as serum and saline are concerned. The pipette, which consists of a length of quill tube drawn out to a capillary end, is actuated by an indiarubber teat; the pipette must always be held vertically and the drops must be delivered slowly (at the rate of about 1 drop

FIG. 24.—Use of Pipette.

per second). Before passing from one reagent to another the pipette must be well washed out and either dried with alcohol and ether or rinsed out with the reagent to be measured by taking up and rejecting the latter several times. (When pipettes have to be interchanged, Donald's methods of standardising should be used.)

The Capillary Pipette Method.[1]—The serum is drawn up in a capillary tube (a piece of quill glass tubing drawn out in the flame is convenient for the purpose) and a mark is made at the upper limit of the fluid, the latter then being blown out in a watch-glass. Equal amounts of 0·85 per cent. salt solution are measured out with the marked tube and added till the dilution necessary is made.

[1] For Wright's methods of measurement, reference should be made to his work on the *Technique of the Teat and Capillary Glass Tube*, London, 1921.

Thorough mixture is effected by drawing up the diluted serum in the quill tube and blowing out again, this being repeated several times.

Agglutination.—By *agglutination* is meant the aggregation into clumps of uniformly disposed bacteria in a fluid. At first the small clumps can only be observed with the microscope; when larger they can be seen at low magnifications and finally they become visible to the naked eye. Such clumps when sufficiently large, sediment, and the supernatant fluid becomes more or less clear when the fluid is allowed to stand. The blood serum may acquire this clumping power towards a particular organism under certain conditions—these being chiefly met with when the individual is suffering from the disease produced by the organism, or has recovered from it, or artificially as the result of injections of the organism. The nature of this property will be discussed later. Here we shall only give the technique by which the presence or absence of the property may be tested. The essential process is the bringing of the diluted serum into contact with the bacteria uniformly disposed in a fluid. The mixture is placed in a small tube; sedimentation is shown by the formation within a given time (say from two hours at 55° C. to twenty-four hours at room temperature) of a deposit at the bottom, the fluid above being clear. If the result is watched under the microscope in a hanging-drop preparation, the occurrence of the phenomenon is shown by the aggregation of the bacteria into clumps, and if the organism is motile this change is preceded or accompanied by more or less complete loss of motility. Two points should be attended to: (*a*) controls should always be made with normal serum and with the bacterial emulsion alone, and (*b*) the serum to be tested should never be brought in the undiluted condition into contact with the bacteria. The stages of procedure are the following:

Blood having been obtained and the serum separated (for methods see pp. 149, 165)—

1. The serum may be diluted (*a*) by means of a graduated pipette or by the drop method. In this way successive dilutions can be rapidly made in $3 \times \frac{1}{2}$ inch test-tubes. In general, a series of doubling dilutions is suitable, *e.g.* 1 : 5, 1 : 10, 1 : 20, etc., though, of course, any relation between the successive dilutions may be selected. This is the best method. (*b*) By means of a capillary pipette.

2. The bacteria to be tested should be taken from young, well-grown cultures, preferably not more than twenty-four hours old, incubated at 37° C. They may be used either as a broth culture or as an emulsion made by adding a small portion of an agar culture to broth or 0·85 per cent. solution of sodium chloride. In the latter case successive small amounts of saline are added to the mass of bacteria, and suspension is aided by gently rubbing the culture

with a looped wire. When a thick turbidity is thus obtained, the suspension is allowed to stand for half an hour at 37° C. to let gross particles settle out, and then the organisms should be uniformly mixed with the rest of the fluid. The bacterial emulsion ought to be distinctly turbid. In the case of organisms of the enteric group, 3 to 5 c.c. fluid are used to suspend one agar culture, so as to give a density of 1000–2000 million organisms per c.c. as judged by the opacity (see p.159). If living organisms are used, precautions must be taken against accidental infection. For most purposes suspensions killed by exposure at 56° C. for half an hour are suitable, or killing may be effected by using saline to which 1 : 1000 formalin has been added. For methods of distinguishing flagellar and somatic agglutination, see p. 141. In the case of rough variants, which tend to sediment spontaneously in physiological saline, the reduction of the NaCl content to 0·4–0·1 per cent often yields a stable suspension. With streptococci the nature of the culture medium on which the organisms have grown is important (p. 293). Shaking the suspension in a vessel with glass beads, may help to render it homogeneous.

3. For the *naked-eye* test, mix equal parts of diluted serum and of bacterial emulsion, and place in an agglutination tube or a narrow test-tube ($\frac{1}{8}$ inch diameter). Keep in the upright position for twenty-four hours at room temperature, at 37° C. for four hours, or at 55° C. in a water bath for two hours (the convection currents set up when the tubes are only partially immersed hasten the appearance of flocculation). It is important to observe not merely the occurrence of agglutination, but also the weakest concentration of the serum with which the reaction can be obtained. Thus if the highest dilution of the serum which produces agglutination is 1 : 8000, this is expressed by saying that 8000 is the " titre " of the serum. The details of the procedure are as follows in the case of testing a patient's serum with *B. typhosus*. A series of small ($3 \times \frac{1}{2}$ inch) test-tubes, *e.g.* six, is set up. Into each of the tubes with the exception of No. 1, 0·4 c.c. saline is measured. Then to 0·1 c.c. serum there is added 1·4 c.c. saline, thus making a 1 : 15 dilution ; of this dilution 0·4 c.c. is added to tubes Nos. 1 and 2, and the contents of the latter are mixed by drawing up into the pipette several times. From No. 2, 0·4 c.c. is transferred to No. 3, and, after mixing, 0·4 c.c. from the latter is transferred to No. 4 and so on, up to tube 5, 0·4 c.c. from No. 5 being rejected. The result is that tubes Nos. 1 to 5 each contains 0·4 c.c. of the following dilutions of serum—1 : 15, 1 : 30, 1 : 60, 1 : 120, 1 : 240 ; tube No. 6 contains no serum. Now 0·4 c c. of bacterial suspension is added to each tube and the contents are mixed and transferred by means of a capillary pipette to the agglutination tubes, beginning with No. 6 and proceeding backward in order to No. 1. Thus the bacteria are exposed to the following concentrations of serum—1 : 30, 1 : 60, 1 : 120, 1 : 240, 1 : 480. The results are best read with the naked eye by holding the tubes against a dark background, with a bright light in front of the observer. The naked-eye method of testing for agglutination is much preferable to the microscopic, as the results with the former are more accurate.

In the *microscopic* form of the test equal quantities of the diluted serum and the bacterial emulsion are mixed on a glass slide, covered with a cover-glass, and examined under the $\frac{1}{6}$-in. objective. The

form of glass slide used for hanging-drop cultures (Fig. 12) will be found very suitable. The ultimate dilutions of the serum will, of course, be double the original dilutions.

Flocculation on a slide is a useful method for preliminary observations, *e.g.* for testing suspicious colonies from plate cultures of fæces in order to determine which should be submitted to further examination. Drops of a low dilution of agglutinating serum (*e.g.* 1 : 100 of an antiserum with a titre of 5000) are placed on a slide and a loopful of organisms from each colony is rubbed up with the wire in successive drops, so as to yield a very dense turbidity; then the slide is rocked vigorously for a minute or so. If flocculation occurs, obvious granularity to the naked eye develops.

Standard Agglutinable Cultures.—Since different cultures of the same organisms (and even different subcultures of a single strain) may differ in their agglutinability by the same antiserum, Dreyer has devised a method of standardising such cultures. The organism (e.g. *B. typhosus*) is grown for twenty-four hours at 37° C. in ordinary veal peptone broth in an Erlenmeyer flask. At the end of this time the flask is shaken, and there is added to it 0·1 per cent. of commercial formalin; it is again shaken and placed at once in a cool chamber at about 2° C. in the dark. The shaking is repeated at intervals for four to five days, the flask always being replaced in the cold chamber. At the end of three or four days the culture will be found to be sterile and will keep practically indefinitely. Such killed cultures are very suitable for sedimentation tests. When a new specimen of culture is to be employed, it is tested for agglutination with varying dilutions of an antiserum, so as to determine the titre of the latter. Simultaneously, the titre of the same antiserum is determined when used along with the standard culture. If the two titres differ, then the former divided by the latter yields a factor which must be employed to divide the titres of all sera tested with the new culture in order to make them comparable with results obtained with the standard culture. In this way successive batches of agglutinable culture are compared and the factor for each is determined.[1] Accordingly where the agglutinability of a batch of bacterial suspension is not standard, the "standard" titre is gained by dividing the actual titre (obtained as described below) by the suspension factor which is supplied.

In testing a patient's serum Dreyer recommends preparing in agglutination tubes by the drop method a series of dilutions and a control without serum as follows:

	Tube 1	2	3	4	5
Drops of saline . .	0	5	8	9	10
1/10 serum . . .	10	5	2	1	0
Standard culture . .	15	15	15	15	15
Final dilution = . .	1/25	1/50	1/125	1/250	Control.

The mixtures are kept at 50°–55° C. for two hours in the case of the enteric organisms (four hours in the case of dysentery bacilli)

[1] These agglutinable cultures are supplied by the Standards Laboratory, University of Oxford.

and the results are read after the tubes have stood for fifteen minutes further at room temperature. "Standard agglutination" represents flocculi just visible to the naked eye.[1] (For further details of this method reference should be made to the Medical Research Council's Special Report Series, No. 51.)

Flagellar and Somatic Agglutination.—Motile bacilli of the normal type of culture of the coli-typhoid group may be agglutinated by antisera acting on the H antigen which is present chiefly in the flagella. The result is rapid formation of large flakes which settle quickly to form a voluminous sediment. On the other hand, antisera which act only on the somatic (O) antigen lead to slow formation of small closely packed granules.

In certain cases of typhoid and paratyphoid infections, it may be important to determine which types of agglutinin are present in the serum, and for this purpose "qualitative receptor analysis" is carried out (Felix). Strains of organisms rich in the respective antigens are selected (*B. typhosus* strain 901 H and O variants, etc., obtainable from the National Collection of Type Cultures), and twenty-four-hours' growths are employed. The agar medium used should be prepared from fresh meat (not meat extract), react neutral to litmus, have abundant water of condensation, and be sterilised at 100° C., as these conditions promote the formation of H antigen. The state of the cultures must be controlled from time to time (*a*) with known anti-H and anti-O sera from rabbits, to determine the presence of the respective antigens, and (*b*) with hypertonic (3·4 and 6·8 per cent.) NaCl solution in which the organisms should form a permanent suspension; sedimentation would indicate change to the rough state, which is unsuitable.

Suspensions of living organisms are best, but failing these, preserved suspensions prepared with phenol or formalin may be used for detection of H agglutinin and one with alcohol for O agglutinin. The latter is prepared by making a suspension of the culture in saline with a density of 3500 to 5500 million organisms per c.c. and adding gradually half its volume of absolute alcohol, with continuous stirring. After the mixture has stood for twelve to twenty-four hours at 37° C. in a capped cylinder, the supernatant suspension is decanted for use; it keeps indefinitely. Before use it is diluted 1 : 6 with saline; the final opacity of the suspension should be two or three times that of the Oxford Standard Agglutinable Culture.

The titre of the serum may then be determined in the usual way by the naked-eye method. In the case of living suspensions the results are read with a × 10 magnifier after two hours at 37° C., and again after sixteen to eighteen hours further at room temperature, since small flake agglutination occurs slowly. With alcohol-treated suspensions twenty-four hours at 52° C. should be allowed. For practical purposes a single dilution of serum (1 : 1000) usually suffices.

The Absorption Method of testing Agglutinins.—This method is applied when several agglutinins acting on allied organisms are present in a serum. The principle is to remove

[1] Control tubes showing standard agglutination for enteric and dysentery bacilli are obtainable from the Oxford Laboratories.

all the agglutinins acting on one organism, and to study the properties of those which remain. In practice, the method consists in adding to a suitable dilution of the serum (*e.g.* a serum whose titre was 5000 would be diluted ten times) an equal volume of a thick emulsion of the bacterium (the organisms of one agar slope being used for 2 c.c. of diluted serum), allowing the mixture to stand at 37° C. for two or three hours, or in the ice-chest overnight, and then separating the bacteria with the centrifuge. The supernatant clear fluid is now pipetted off, and its agglutinating properties studied on the other members of the bacterial group and also, by way of control, on the organism used for absorbing it, in order to make sure that all agglutinins for the latter have been removed. The object of the method is to determine which member of a bacterial group is causally related to the condition from which the serum is obtained, and examples of its application for this purpose will be found in the chapter on typhoid fever (p. 530). Here the principle is that when an unknown strain belonging to such a bacterial group is under investigation, if its capacities for absorbing agglutinins from a serum are the same as those of an already recognised strain, then the two are probably identical. On the other hand, an allied strain to the organism by which the agglutinin has been produced will absorb only part of the agglutinin. Similarly, agglutinin for particular antigenic constituents of an organism may be obtained by the absorption method. Thus in the case of *B. typhosus* an agglutinin for the flagellar antigen may be prepared from an antiserum containing both this and the somatic agglutinins, by absorbing the latter with a suspension of *B. typhosus* of a variant strain containing only the somatic antigen; or a boiled suspension of an ordinary strain may be used for the purpose.

Vi Agglutination.—In the case of certain smooth strains of *B. typhosus* and members of the Salmonella group, the bacilli in the living virulent state are inagglutinable by a pure homologous anti-O serum. These organisms are agglutinated by an antiserum which has been developed in rabbits by injection of bacteria which contain the Vi antigen—the H and O agglutinins having been absorbed from the serum by previous treatment with cultures containing only the latter antigens. The agglutinated organisms form fine granules.

Method of obtaining Agglutinating Sera.—For the identification of organisms by their agglutination reactions artificial antisera of high titre must always be employed (human sera from cases of natural infection should never be used). Rabbits are in general most suited for developing specific antisera; old animals ought to be avoided. Cultures killed by heat are generally effective, as low

a temperature as possible being employed, *e.g.* half to one hour at 56° C. Intravenous injection is as a rule to be preferred, a small dose being given at first (1/50 of a killed twenty-four-hours' agar-slope culture suspended in saline), followed by gradually increasing doses (each being double the preceding one) at intervals of seven to ten days. (Formalin 1 : 1000 or other antiseptics may be used to kill the organisms; also a broth culture may be employed, the initial dose being 0·5 c.c.). During the course of immunisation the animal should be weighed frequently. Loss of weight suggests that the dosage is too high; if the animal's health is seriously upset, powerful antisera will probably not be obtained. But it must be remembered that some animals respond to the injections by more active production of antibodies than others. In the case of highly toxic organisms, such as Shiga's dysentery bacilli, a smaller initial dose is used, *e.g.* 1 . 100 of a culture, or their toxicity may be diminished by heating at 80° C. for an hour. Seven to ten days after the second or third dose a specimen of blood may be withdrawn from the ear vein and tested. If the titre is high (2000 or more) the animal may be bled; 20 c.c. of blood may be obtained from the ear vein, and this may be repeated on several successive days provided the animal is supplied with abundance of water to drink Or the animal may be anæsthetised with ether and bled from the carotid artery. The blood is received into a sterile tube which has been rinsed out with saline to ensure retraction of the clot The serum which has separated from the clot on standing for twenty-four hours in a cool place is stored in lengths of sterile quill-tubing drawn out at the ends, or in ampoules which are sealed in the Bunsen flame. It may be preserved by heating for half an hour at 56° C. several times, or by the addition of an antiseptic (p. 913) and kept in the ice-chest; or the serum, without any treatment or addition, may be kept frozen.

Opsonic Methods

Method of measuring the Phagocytic Capacity of the Leucocytes.—This was first done by Leishman by a very simple method, as follows:

Equal quantities of blood and of a fine emulsion of the bacterium to be tested are mixed together, a small drop of the mixture is placed on a glass slide and covered with a cover-glass; the preparation is placed in the incubator at 37° C. for fifteen minutes. The cover-glass is then slipped off and the film on the slide stained by Leishman's method. A control preparation can be made with normal blood in the same way, and the two films are stained as one. The number of bacteria present in, say, fifty polymorphonuclear cells successively examined is determined, and an average struck.

Since phagocytosis depends upon certain substances in the serum to which Wright gave the name *opsonins* (p. 229), he elaborated the following method by which its degree could be estimated:

(1) *Preparation of Bacterial Suspension.*—In the case of organisms such as the pyogenic cocci, some of a twenty-four-hours' living culture

on a sloped agar tube is taken and rubbed up with 0·85 per cent. saline so as to obtain a suspension consisting of single bacterial cells. With certain organisms, *e.g.* streptococci in chains, a good deal of trituration may be necessary, and often centrifuging must be practised, for the removal of clumps. Only by experience can a knowledge be gained of the amount of culture to be used in the first instance, but the resultant suspension usually should exhibit only the merest trace of cloudiness to the naked eye. If too strong a suspension be used, the leucocytes may take up so many organisms that these cannot be accurately enumerated. When intensely pathogenic organisms are used—*e.g. B. pestis, B. melitensis*—Wright recommends that the culture should be first killed by suspending in 40 per cent. formalin. The latter is then removed by centrifuging and the deposit washed with saline. In the case of the tubercle bacillus, Wright directs that a seven- to ten-day culture in glycerol broth should be sterilised by heat, collected on a filter, washed with salt solution, and dried. Ten milligrams of the dry culture should be powdered in a small agate mortar, a drop of 1 per cent. saline added, and the sticky paste triturated for about five minutes; further saline is added drop by drop till a thick suspension is obtained of the bulk of about 1 c.c. This is centrifuged and the supernatant suspension pipetted off and diluted to the necessary degree.

(2) *Preparation of Leucocytes.*—Here the observer uses his own blood cells. A 1·0 per cent. solution of sodium citrate in 0·6 per cent. sodium chloride is prepared. This is placed in a centrifuge tube, which is filled nearly to the brim. A tape being wound round the finger, this is now pricked, and the blood allowed to flow directly into the fluid, to the bottom of which it sinks. The tube ought to be inverted between the addition of every few drops of blood, so as to bring the blood in contact with the citrate and prevent coagulation. The equivalent of about 10 to 20 drops of blood should be obtained. The diluted blood is then centrifuged, and when the corpuscles are separated the supernatant fluid is removed, fresh saline is substituted, and the centrifuging repeated. A second washing with saline is practised, the supernatant fluid removed, and the greyish surface layer of blood, which is rich in leucocytes, removed by a fine pipette. The leucocytes may be thoroughly mixed by drawing up in a fine pipette and blowing out again, this being repeated several times.

(3) *Preparation of the Sera.*—Each sample of serum is prepared by the methods described on p. 165. In every case serum from a normal individual should be prepared as a control.

The suspension, corpuscles, and serum being thus prepared, an equal quantity of each is taken by a small capillary pipette, and a thorough mixture is made in the usual way. A small portion of the mixture is taken up in a capillary tube, and its ends are sealed by heat, care being taken that the contents are not over-heated. The tube is then placed in the incubator at 37° C. for fifteen minutes. At the end of this time a drop of the mixture is placed on a slide, and a film preparation is made by spreading out slowly with the edge of another slide, so that a thick margin is formed at the end; here the majority of the leucocytes collect. In the case of ordinary bacteria the film is stained by Leishman's method. With tubercle bacilli the following is the procedure: The film is fixed, washed thoroughly, stained with carbol-fuchsin as usual, decolorised with

2·5 per cent. sulphuric acid, cleared with 4 per cent. acetic acid, washed with water and counter-stained with watery solution of methylene-blue (to which ½ per cent. sodium carbonate may be added), and dried.

The two preparations are now examined microscopically with a movable stage, the number of bacteria in the protoplasm of at least a hundred polymorphonuclear leucocytes is counted, and an average per leucocyte struck ; the proportion which this average in the case of the abnormal serum bears to the average in the preparation in which the healthy serum was used, constitutes the *opsonic index*—that of healthy serum being reckoned as unity.

In the case of such organisms as those of the coli-typhoid group and cholera, which are susceptible to bacteriolytic influences in the serum, it may be necessary to heat the sera of the patient and observer for half an hour at 55° C. This destroys any complement present and prevents bacteriolysis occurring. In the case of the *B. typhosus* the virulence of the strain employed has been shown to be an important factor.

Several modifications of Wright's technique have been suggested. For example, a series of mixtures may be made in which the serum is added in increasing dilutions. The highest dilution of each serum is ascertained with which 100 leucocytes contain about 50 organisms. The ratio of this limiting dilution for the patient's serum to that with the control serum yields a measure of the opsonising power of the former. Simon compares not the numbers of bacteria ingested, but the percentages of cells containing bacteria and those not containing bacteria. This he calls the "percentage index," and he states that the figure thus obtained corresponds very closely to the ordinary opsonic index ; he claims that the method eliminates some of the errors which may arise in the use of the ordinary technique if only a relatively small number of phagocytosing cells, such as fifty, be examined.

Bactericidal Methods

The Estimation of the Bactericidal Action of Serum.—This may be carried out by various methods, of which those of Neisser and Wechsberg and of Wright may be given as examples. In the former, the effects of varying amounts of serum on the same amounts of bacteria are observed by means of plate cultures ; in the latter, the number of bacteria which can be completely killed off by a given quantity of serum is ascertained. In carrying out experiments of this kind it is convenient to have a number of small test-tubes sterilised and plugged with cotton wool. We can then make any required dilution of a young broth culture (or instead a suspension of a culture on solid medium) as follows : To each of a number of tubes add 0·9 c.c. of some neutral diluent such as Locke's solution containing 0·5–0·7 per cent. gelatin and adjusted to a pH of 7·5 with sodium bicarbonate. To the first tube (*a*) we add 0·1 c.c. of the

bacterial culture, and thoroughly shake up the mixture ; to the second (*b*) we add 0·1 c.c. of the contents of (*a*), and shake up ; to the third tube (*c*) we add 0·1 c.c. of the contents of (*b*), and so on. It is thus evident that 0·1 c.c. of the contents of (*a*) will correspond to 0·01 c.c., and 0·1 c.c. of (*b*) to 0·001 c.c. of the original culture ; any required fraction can thus be readily obtained. In the making of the mixtures it is essential that none of the bacteria shall escape the action of the serum, *e.g.* by remaining on a part of the mixing vessel with which the serum does not come in contact.

(*a*) *Method of Neisser and Wechsberg.*—A series of small plugged sterile tubes is taken, and to each is added 0·5 c.c. of neutral diluent,[1] and a given quantity, say 0·0002 c.c., of a young broth culture to be tested. To the several tubes in series we then add varying amounts of the fresh serum whose action is to be observed, *e.g.* 0·2 c.c., 0·1 c.c., 0·05 c.c., 0·025 c.c., etc. The contents of each tube are then made up to 1 c.c. with diluent, and a few drops of sterile broth are added to each tube. The tubes are then well shaken and placed in the incubator at 37° C. for three hours, to allow the serum to act. (Of course several series of such tubes may be prepared and placed in the incubator for varying periods of time ; we can thus observe when the bactericidal effect reaches the maximum.) At the end of the given period of time a small quantity, say 0·05 c.c., of the contents of each tube is added to a tube of melted agar (cooled to about 45° C.) ; the contents of each agar tube are then mixed and poured out into a sterile Petri capsule The Petri capsules are placed in the incubator for a suitable period of time. The number of colonies in each can then be noted. Of course gelatin can be substituted for the agar in the plates if desired.

(*b*) *Wright's Method.*—A twenty-four-hours' broth culture is used, and various dilutions with sterile broth are made according to the method described above; thus 5-, 10-, 20-, 50-, 100-, 1000-, etc., fold dilutions may be prepared. A small quantity, say 1 c.mm., of the fresh serum to be tested is mixed with an equal amount of the bacterial culture, and the mixture is placed in a small capillary tube which is sealed at the ends ; similar mixtures of equal parts of serum and of each of the dilutions of culture are prepared and treated in the same way. The tubes are then placed in the incubator for eighteen to twenty-four hours at 37° C., and at the end of that time the contents of each are tested as regards sterility by means of cultures. In this way the greatest dilution in which the bacteria are completely killed off is ascertained. The number of bacteria in the original culture per c.mm. can be counted by the method given on p. 91, and thus the total number of bacteria killed by the quantity of serum used can be readily calculated.

(*c*) The following method used by *Mackie and Finkelstein* can be carried out rapidly and gives useful results, *e.g.* with normal serum. A series of six or more tubes containing 0·5 c.c. serum are

[1] 0·85 per cent. NaCl solution was used originally ; but some organisms *e.g.* pneumococci or gonococci, die quickly in salt solution.

each inoculated with 0·1 c.c. of bacterial suspension of varying density—it is convenient to use decimal dilutions, *e g.* 1 : 100, 1 : 1000, etc., of a standard suspension of a twenty-four-hours' culture. In making these dilutions the pipette should be changed at each step. With a standard loop a single stroke is made at once from each of the mixtures on a plate of medium (a 4-inch plate will accommodate twelve such strokes, which should all be of equal length). A suitable series of dilutions will yield only scanty colonies or no growth in the stroke from the highest dilution. After incubation at 37° C. for four hours or longer another similar plate is inoculated in the same way from each tube. After time has been permitted for growth to occur on the plates at 37° C. the results are compared. Bactericidal action is shown by the occurrence on the second plate of only scanty colonies or no growth in a series of concentrations higher than those yielding the same result on the original plate. The approximate degree of bactericidal action is determined by the difference between the end points of growth in the series of stroke cultures before and after incubation.

The *bactericidal action of whole-blood* can be estimated by the same method, defibrinated or preferably heparinised blood being tested in place of serum. The mixtures must be shaken repeatedly during incubation to prevent sedimentation of the blood cells. If the incubation is prolonged some form of shaking machine is required which will agitate the tubes just sufficiently to maintain the blood in suspension (Todd) (for details of these methods see the original papers of Mackie and Finkelstein). Other methods have been used by Wright, and Robertson and Sia.

(*d*) As will afterwards be described in greater detail (*see* Chapter on Immunity), when an animal is immunised against a particular bacterium the bactericidal action of its serum may be greatly increased, and this depends on the development of a particular substance called an immune-body, which is comparatively thermostable and is not destroyed at 55° C. To analyse the bactericidal properties of such a serum, it should in the first place be heated in order to destroy the normal complement. Then to each of a series of sterile tubes we add (*a*) a quantity of normal unheated serum insufficient of itself to destroy the bacteria, to act as complement, (*b*) a given amount of the bacterial culture, and (*c*) varying amounts of the heated immune-serum — 0·1, 0·01, 0·001, etc., c.c. The mixtures are then incubated and a fixed amount of each is plated, as described for Neisser and Wechsberg's method. In this way we can find the quantity of the immune-serum which gives the maximum bactericidal action.

Fixation of Complement.

In some cases when an animal is immunised against a given bacterium, or when a patient is infected with the organism, the serum may not have increased bactericidal action, but nevertheless contains an immune-body which leads to the absorption or fixation of complement. In other words, the immune-body is a substance which, along with the corresponding or homologous bacterium, binds complement (p. 224). In

order, however, to explain the methods by which the fixation of complement may be demonstrated, we must first of all give some facts with regard to hæmolytic sera.

Methods of Hæmolytic Tests.—A hæmolytic serum is usually prepared by injecting the red corpuscles of an animal into an animal of different species, *e.g.* intraperitoneally ; the corpuscles of the ox or sheep are most frequently used, and the rabbit is the most suitable animal for injection. The corpuscles ought to be completely freed from serum by repeatedly washing them in sterile salt solution, and centrifuging. An intraperitoneal injection of the corpuscles of 5 c.c. of, say, ox's blood, followed by two injections, each of 10 c.c., at intervals of eight days, will usually give an active serum. About a week after the last injection a specimen of blood should be taken from the ear and its titre estimated. If this is satisfactory, the animal should be anæsthetised and bled, *e.g.* by cardiac puncture. The serum which separates (after centrifuging to remove any suspended red corpuscles) may be collected in suitable lengths of quill glass-tubing drawn out at the ends, which are afterwards sealed in the flame. To ensure sterility when the serum is to be kept some time, it is advisable to heat it for an hour at 55° C. on three successive days ; we have always found that serum treated in this way remains sterile. It is, of course, devoid of complement. The test amount of corpuscles is usually 0·5 c.c. of a 5 per cent. suspension of blood in 0·85 per cent. sodium chloride solution ; that is, the corpuscles of 5 c.c. blood are completely freed of serum by repeatedly washing in salt solution, and then salt solution is added to make up 100 c.c. (or 3 c.c. of tightly packed blood sediment *plus* 97 c.c. saline). In any investigation it is necessary to ascertain the minimum hæmolytic dose (M.H.D.) of the immune-body and of the complement to be used. (It is to be noted that as complement does not increase during immunisation, the hæmolytic dose of the fresh serum will come far short of representing the amount of immune-body present.) In testing the *dose of immune-body,* the fresh serum to be used as complement must be devoid of hæmolytic action (in the present instance rabbit's serum will be found suitable), and more than sufficient to produce lysis with immune-body is added to each of a series of tubes. Instead of rabbit's serum it is preferable to use guinea-pig's serum as the source of complement, since it is much more active. The latter often contains some natural immune-body for ox's red corpuscles, which may be removed by the following procedure. Equal volumes of guinea-pig's serum and of sharply centrifuged washed ox blood sediment in

separate tubes are chilled by immersion for five minutes in chopped ice, then they are mixed and the mixture is kept on ice for an hour. The treated serum is recovered by centrifuging and is pipetted off; it is now devoid of any lytic action by itself, but contains the complement; 0·05 c.c. of this treated serum is added to each tube in estimating the dose of immune-body. Varying amounts of immune-body (which should be diluted with saline in order to permit of accurate measurement) are added to the tubes, the contents are shaken, and incubated at 37° C. for one and a half hours. The amount of lysis is then noted. The smallest amount of immune-body which gives complete lysis is, of course, the M.H.D.; this may be as low as 0·0005 c.c. for the test amount of corpuscles. An immune-body for sheep's red corpuscles can be developed similarly by injections of washed sheep's blood. Since guinea-pigs' serum seldom has much natural hæmolytic action on these corpuscles it can be used without previous treatment with the blood at 0° C.

To estimate *the M.H.D. of complement*, proceed in a corresponding manner; to each of a series of tubes add several (at least five) doses of immune-body, and then to the several tubes different amounts of complement. Nearly maximal lysis is obtained after one hour's incubation. The amount of complement necessary for lysis varies somewhat according to the amount of immune-body used, being smaller with several doses of the latter than with a single dose; in estimations of the dose of complement, it is accordingly advisable to use the optimum amount of immune-body, in the present instance about five hæmolytic doses. The activity of a serum as complement varies considerably, and each sample must be separately tested.[1] Corpuscles treated with sufficient immune-body to produce complete lysis on the addition of complement are usually spoken of as *sensitised corpuscles*.

The above will serve as an indication of the fundamental methods.

The Removal of Blood-Samples from Rabbits.—In such work as that just described, it is often convenient to watch the progress of an immunisation procedure by removing a sample of blood

[1] Complement rapidly (often within twenty-four hours) loses its strength when kept at room temperature. It can, however, be preserved for a considerable time at or near its original strength if it be kept frozen. Even if this be done, however, the strength of the complementing serum must be titrated at the commencement of every experiment in which it is employed. Commonly the M.H.D. of fresh guinea-pig's complement for 0·5 c.c. ox or sheep corpuscle suspension is 0·005 or 0·0075 c.c.

without the animal being killed. With proper care any amount of blood up to one-third of that contained in the body can be removed from the ear vein of a rabbit. The animal, which must not be excited, is placed on a bench, and its body kept warm by being covered with a cloth. The root of the ear should be shaved over the marginal vein, the hairs on the edge of the ear should also be clipped short. It is best to have the ear dry, as the evaporation of a fluid causes contraction of the vessels. In a great deal of hæmolytic work absolute sterility of the sample is not necessary, so that washing the ear is not required. When sterile blood is desired, the precautions detailed on p. 68 may be applied. A frosted incandescent electric lamp, such as is used for microscopic illumination, is placed lighted an inch or two from the ear. The left hand of the operator should cover the animal's head in front of the ears, the thumb and index-finger being left free to compress the vein at the root of the ear. In this way not only is the animal's eye protected from the glare of the lamp, but the distance of the latter from the ear can be regulated so as to keep it at what to the operator's hand is a pleasant warmth. In a minute or two the ear vessels will dilate, and the vein, being compressed at the root, a lateral opening is made with a bayonet-pointed surgical needle (the triangular-pointed needles supplied with the Gowers-Haldane hæmoglobinometer are also very suitable), and the blood allowed to drop into a sterile test-tube. Usually waves of contraction of the ear vessels will be observed to occur, the passing off of which must be waited for, and from time to time the clot must be gently squeezed out of the opening in the vein with the flat side of the needle, or it may be necessary slightly to enlarge the opening. The blood should be allowed to clot completely, and then, by means of a sterile platinum wire, the clot should be loosened from the sides of the tube in order that it may freely contract. The tube should be placed in the ice-chest till the following morning, when the serum can be pipetted off with a sterile pipette. After a considerable amount of blood has been withdrawn, the animal should be freely provided with water for drinking.

Daily samples can thus be obtained from an animal. If care be taken not to make ragged openings in the vein, often the simple removal of the previous scab will be followed by a free blood flow.

This method can be applied in guinea-pigs, provided these be of fair size. Here successive samples of 2 c.c. can be obtained from the ear veins.

Guinea-pig Complement.—The animal is bled by severing the neck vessels over a large glass funnel (6 inch diameter) which dips into a measuring cylinder—the glass-ware is sterilised beforehand by heat, and when cool is rinsed with normal saline. After the blood has coagulated, the clot is detached from the glass by means of a sterile wire in order to aid separation of the serum. It is usually advisable to pool the sera of several animals. The complement can be preserved for several months in the frozen state at a temperature below $-10°$ C. Complement which has been dried from the frozen state *in vacuo* (Craigie), also furnishes a stable reagent which keeps well; before use it is dissolved in sufficient distilled water to make up the original volume.

Complement Fixation Reaction of Bordet and Gengou.—From the facts given above it follows that sensitised corpuscles, *i.e.* corpuscles treated with immune-body, may be made to serve as an indicator for the presence of complement. If an antibacterial immune-body is present in a serum heated at 55° C., the serum when added to the corresponding bacterium leads to the fixation of complement, and thus prevents hæmolysis when sensitised corpuscles are added. If we represent the bacteria, or rather the receptors in the bacteria, by X, the immune-body by anti-X, and the complement by C (normal serum, say, of a guinea-pig) we may represent the method of experiment by the following scheme:

$$X + \text{anti-}X + C \;\vdots\; + \text{sensitised corpuscles} \;\vdots$$

(The vertical dotted line represents a period of incubation for one and a half hours at 37° C.)

If lysis of the sensitised corpuscles does not occur after incubation at 37° C., then the complement has been fixed and an immune-body has been shown to be present, provided that a suitable control shows that the bacteria alone, without immune-body, do not fix sufficient complement to interfere with lysis. The reaction may also be controlled by substituting for the immune serum heated normal serum of the same species; absence of complement-fixation under these conditions shows that there is no natural antibody present.

This method has now been extensively used for demonstrating the presence of immune-bodies in the blood of patients suffering from a particular bacterial infection. It has also been applied to determine whether a suspected bacterium is really the cause of a disease, for if the bacterium gives with the serum of the patient fixation of complement, then there is a strong presumption that it is the infective agent (*vide* Immunity). The method as carried out is practically the same as that of the Wassermann reaction (see below) except that the antigen consists of broth cultures or suspensions in saline of slope cultures of the organisms, which have usually been killed by heating. The optimum amount of organisms to be employed must be ascertained in every instance, but the test amount of bacterial suspension should not by itself inhibit more than one or two doses of complement. It is essential that antisera which are to be tested in this way should not have been obtained from animals injected with cultures grown on foreign blood or serum, since antibodies generated by the latter may lead to fallacies.

Complement fixation may be said to occur when the mixture of antigen and serum inhibits more complement than the sum of the amounts inhibited by each of these reagents separately; but the phenomenon is clearly present when the mixture of antigen with the serum of an individual infected or inoculated with the organism in question inhibits complement to a greater degree than does a similar mixture containing serum from an uninfected individual.

In carrying out complement fixation tests care must also be taken that the source of complement, usually guinea-pig's serum, does not contain a natural antibody for the antigen employed. In order to make sure of this a control is included in the series, in which the effect on complement of the antigen by itself is determined. It has been found in the case of *B. typhosus* and other organisms that under such conditions a small amount of complement may fail to be fixed, whereas fixation occurs in the tubes containing larger amounts (Dunlop). This disturbing property of the guinea-pig's serum may usually be removed without damaging the complement by keeping it at 37° C. for several hours or by treatment with charcoal.

The Serum Diagnosis of Syphilis—Wassermann Reaction

Wassermann, Neisser and Bruck, proceeding in accordance with the facts established with regard to the fixation of complement, tested whether a similar phenomenon might not be obtained in the case of syphilis. They mixed together a watery extract of congenital syphilitic liver, rich in spirochætes (antigen), and serum from a syphilitic case (supposed to contain antibodies), and found that a relatively large amount of complement was fixed. On the other hand, when the serum from a non-syphilitic case was substituted for the syphilitic serum, little or no fixation of complement occurred. The result was thus in accordance with expectations on theoretical grounds. Marie and Levaditi, however, found that an extract of normal guinea-pig's liver along with syphilitic serum fixed complement, *i.e.* acted as antigen. Subsequent observations (Porges and Meier and others) showed that alcoholic extracts of normal as well as syphilitic tissues are also very efficient, and that the property resides mainly in the fraction of the extract which is soluble in alcohol and ether but insoluble in acetone (lecithin). The addition of cholesterol to lecithin enhances greatly its

activity (Browning, Cruickshank, and Mackenzie). Although abundant observations have established the validity of the test as a means of diagnosis, the reaction which led to its discovery is no longer sufficient to explain it.

It must be noted, however, that the substance in the syphilitic serum which leads to the fixation of complement varies greatly in amount in different cases, and in the same case at different times, especially under the influence of anti-syphilitic treatment with salvarsan. Accordingly, it is not possible to state absolutely the quantity of complement which must be fixed in order to give a positive result. Manifestly there will be cases where the amount fixed is just under any standard adopted, and these, which are to be regarded as suspicious or doubtful, will be missed with a one-tube method. Moreover, the amount of complement, as estimated by the hæmolytic dose, varies considerably in different samples of fresh serum; also the complement in different specimens of serum varies in its capacity for being fixed (deviability). It is accordingly necessary for satisfactory results to estimate the hæmolytic dose of the guinea-pig's serum, and to make a series of mixtures, each containing the same amounts of serum and of antigen, but with a different number of doses of complement in each tube. In this way we can find the number of doses of complement fixed in each case. As controls, the effect on doses of complement of the extract alone and of the serum alone should be tested; also, a known negative and positive serum should be tested at the same time (the latter should be one which gives only a weak reaction).

Quantitative Method.—In order to carry out the test, we require (*a*) serum from the suspected case previously heated at 55° C., (*b*) an alcoholic tissue extract (that of human or ox heart, along with cholesterol, being most widely used), (*c*) the fresh serum of a guinea-pig to act as complement, and (*d*) sensitised corpuscles, *i.e.* a suspension of washed sheep or ox corpuscles to which five doses of immune-body have been added. Three tubes with different doses of complement will be sufficient for routine examination. Also, along with each series of tests, the necessary controls must be included (see below).

Outline of the test.—To each of the three tubes add 0·5 c.c. of *diluted antigen* (*vide infra*) and 0·05 c.c. of the serum to be tested (heated for half an hour at 55° C.).

Add to the three tubes respectively, two, four, and six doses of complement—the dose being that for 0·5 c.c. of sensitised corpuscles.

Place the tubes in the incubator for an hour and a half at 37° C.

Then add to each 0·5 c.c. of suspension of corpuscles sensitised

with five doses of immune-body, and place again in the incubator for an hour and a quarter. Place the tubes aside at the room temperature till the non-lysed corpuscles have sedimented—conveniently till next morning—and then read the results

Controls should be made in each test as follows: one tube containing the stated amount of antigen along with two doses of complement, and one containing the stated amount of heated serum along with two doses of complement; to all these 0·5 c.c. of sensitised corpuscles is added after incubation for an hour and a half. It is also essential to put up series with a known negative (preferably a pooled specimen of at least six sera) and a weakly positive serum—in routine work it is convenient to keep such sera from previous tests; they are best preserved by freezing, but if sterile they will keep for a few days in the ice-chest.

Antigen.—Various antigens have been used, but the following gives very satisfactory results:

Human heart muscle from the left ventricle (the cause of death is stated to be immaterial) is freed from visible fat, minced finely, and ground for a minute with absolute alcohol (1 gram of heart to 9 c.c. of alcohol) in a mortar with clean sand. The mixture is shaken in a mechanical shaker for one and a half hours and then filtered. This constitutes heart extract, which keeps at cool room temperature for six months at least. Ox heart extract may be prepared similarly.

Preparation of Diluted Antigen.—1·5 volumes of heart extract are mixed with 1 volume of 1 per cent. cholesterol in absolute alcohol; 1 volume of this solution is mixed rapidly with 29 volumes of normal saline. (It is an advantage in the case of each specimen of extract to test various dilutions of it with a known syphilitic serum and find the dilution which gives most fixation, and then to use this dilution in subsequent tests.)

Practical Performance of Wassermann Test.

Reagents [1].

(*a*) *Patient's Serum* heated at 55° C. for thirty minutes before use (unheated serum from non-syphilitics may react positively).

(*b*) *Antigen Dilution*.—An alcoholic tissue extract (that of human or of ox heart + cholesterol) diluted with saline as described above.

(*c*) *Complement.*—Mixed fresh serum of at least two guinea-pigs (see p. 150). The blood should be drawn and kept overnight (eighteen hours) in the ice-chest; the serum is then separated for use. The complement of serum obtained too short a time after bleeding may be unsuitable, as it tends to be excessively deviable. As a guide to the amounts of complement to be used the M.H.D. is estimated immediately before carrying out the Wassermann reaction as follows: Prepare 1 : 4 dilution of complement (0·3 c.c. saline *plus* 0·1 c.c. guinea-pig's serum); of this add to four tubes each containing 0·5 c.c. sensitised red cell suspension, 0·01, 0·02, 0·03, and 0·04 c.c. respectively. Mix the contents well by shaking and keep the tubes at 37° C. for one hour—shaking at fifteen minute intervals. Take as the M.H.D. the smallest amount of comple-

[1] Use heavy $3 \times \frac{1}{2}$ inch test tubes well cleaned and finally thoroughly rinsed in distilled water to remove traces of acid, alkali, or soap and then dried in the hot-air oven.

ment which causes practically complete lysis; it is usually that in the second or third tube, *i.e.* 0·005 or 0·0075 c.c. of *undiluted complement.*

(*d*) *Sensitised Red Cells.*—Sheep or ox blood suspension +5 M.H.D. of immune-body (p. 148). If the immune serum causes marked agglutination of the red cells, as occurs not infrequently with sheep corpuscles, lysis is interfered with; to avoid this the red cell suspension should be sensitised shortly before use.

Add reagents in the order shown:

	Main Test.			Serum Control.
Tube No.	1	2	3	4
Saline, c.c.	—	—	—	0·5
Antigen dilution, c.c.	0·5	0·5	0·5	—
Patient's serum (55° C.), c.c.	0·05	0·05	0·05	0·05
Complement M.H.D.	2	4	6	2

Mix well the contents of each tube; incubate at 37° C. for one and a half hours, shaking every thirty minutes; then add to each tube 0·5 c.c. sensitised sheep (or ox) red cells; incubate again for one and a quarter hours at 37° C., shaking every fifteen minutes.

Controls for each Series of Tests:

(A) *Antigen Control.*

Tube No.	1	2	3
Antigen dilution, c.c.	0·5	0·5	0·5
Complement M.H.D.	2	4	6

(B) *Complement Control.*

Tube No.	1	2	3	4
Saline, c.c.	0·5	0·5	0·5	0·5
Complement (diluted 1 : 4 with saline), c.c.	0·01	0·02	0·03	0·04

(C) In addition, a known pooled negative and a known weakly positive serum are always tested (see above)

The controls are incubated, etc., along with the main test. Serum and antigen controls show that these reagents separately have only minimal inhibiting effect upon complement. The complement control shows that the complement is active.

Read results after the tubes have stood for some hours at room temperature to allow any unlysed red corpuscles to sediment (see below).

Cerebro-spinal fluid is tested in the same way as serum, except that it is not heated beforehand: 1 volume of the alcoholic antigen is diluted quickly with 29 volumes of the fluid, and 0·5 c.c. of this mixture is measured into each tube. In the control tube, instead of serum and saline, 0·5 c.c. of cerebro-spinal fluid is used.

Reading of Results.—It will usually happen that the test with the antigen alone, with the serum alone, and also that with the mixture of antigen and the negative control serum, show practically complete lysis in the first tube, *i.e.* with two doses of complement. This being the case, the result of the test is decided as follows: (*a*) A serum which shows practically or quite complete lysis with the same amount of complement as causes complete lysis of the negative control serum is *negative.* (*b*) A serum which requires from one and a half times to twice as much complement as does the negative control to produce a given degree of lysis is *suspicious*—recorded as " ? " or " ± ". (*c*) A serum which fixes more complement than is specified under (*b*), but which shows complete lysis with the highest amount of complement, is a *weak positive.* (*d*) A serum which shows no lysis in any tube is a *strong positive, i.e.* one which fixes upwards of five doses of complement more than the sum of the amounts inhibited by the antigen and the patient's serum separately.

The interpretation must vary somewhat according to circumstances; for example, a suspicious or weakly positive reaction would be accepted as positive in the case of a syphilitic patient undergoing treatment, but would not be accepted unconditionally as evidence of syphilis in a case in which the diagnosis was in doubt.[1]

Some observers use the same amount of complement in each tube, but vary the amounts of suspected serum, and in this way an estimate of the deviating power of the serum is obtained. We consider that the method given is to be preferred; but occasionally where the usual amount of patient's serum gives only a doubtful positive result double this amount (0·1 c.c.) may lead to more marked fixation of complement.

Flocculation Reaction.—The occurrence of precipitates when syphilitic sera were mixed with lecithin and other colloids was observed soon after the discovery of the Wassermann reaction, but it is only recently that reliable forms of this test have been devised. The following is a modification of the method of Sachs and Georgi:

Each patient's serum, which should be freshly taken, is heated at 55° C. for half an hour and 0·4 c.c. of each of the following dilutions is prepared in six $3 \times \frac{1}{2}$ inch test tubes—1 : 2, 1 : 4, 1 : 8, 1 : 16, 1 : 32, 1 : 64—and a further tube receives 0·4 c.c. saline (this acts as a control of the antigen emulsion for the series of tests); then to each tube 0·2 c.c. of the antigen emulsion is added. When the

[1] Another method of performing the test is described by Wyler, Medical Research Council, Special Report Series, No. 129, London, 1929.

optimum dilution of the antigen in the test is 1 : 18 a 1 : 6 emulsion is used. The latter is prepared by mixing rapidly equal quantities of the undiluted antigen and saline solution, allowing the mixture to stand for ten minutes and then adding sufficient saline to give the required dilution of 1 : 6. The mixtures of serum and antigen are transferred to agglutination tubes (p. 139), incubated at 37° C. for four hours and allowed to stand overnight at room temperature, when readings are made. The final reading should be made after forty-eight hours. In reading the results the tubes are held in front of a shaded lamp so that they are brightly lit without the rays of the lamp reaching the eyes directly. Strongly reacting sera may cause flocculation in the highest dilutions; with weak sera flocculation may be present only in the tube with the highest amount of serum. The appearances resemble those in bacterial sedimentation. Zone phenomena sometimes occur in which flocculation is less with the higher concentrations of serum than with lower. Known negative and positive sera should be included in every series of tests. In the antigen control and in tests with negative sera no flocculation occurs, the contents of the tubes being homogeneous and slightly opalescent.

Antigen.—This is prepared as follows: 20 grams of sheep's heart muscle, freed from gross fat and fibrous tissue, are ground with sand in a mortar and extracted for four days at room temperature with 100 c.c. of 96 per cent. alcohol. The extract is filtered and 0·25 gram cholesterol is added and the mixture is kept at 37° C. for some days. It is then allowed to stand at room temperature for a day and filtered. This solution keeps indefinitely. In order to standardise a new antigen it must be compared, as regards the sensitiveness of different dilutions, with the optimum dilution of an antigen whose behaviour has been previously tested. For this purpose emulsions are prepared from 1 : 1 cholesterol-extract saline mixture made as described above, by adding to a fixed amount of the latter varying quantities of saline, so as to give emulsions of strengths 1 : 4, 1 : 5, 1 : 6, 1 : 8 of the original undiluted antigen. Each of these emulsions is then tested as described above with a series of positive and negative sera, and a parallel test is carried out at the same time with the same sera and the optimum dilution of the known antigen. A number of such tests are made on several separate occasions. The concentration of the new antigen which is at least as sensitive to positive sera as the optimum of the old and which does not flocculate either alone or with negative sera, is selected as the optimum dilution.[1] (A difficulty of such tests is that the sensitiveness of the antigen emulsion tends to vary in an unpredictable way, *e.g.* on occasions the emulsion will be flocculated by the control negative sera.)

Another method of performing the flocculation test is that described by Mackie and McCartney (see *An Introduction to Practical Bacteriology*, Edinburgh, 1934). Further procedures are given by Meinicke; Dreyer and Ward; Kahn. (See also League of Nations Publications.)

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[1] Krishnan has described in further detail methods of standardising the antigen. *Ind. Journ. Med. Res.* (1929–30), xvii, 477.

The Preparation of Vaccines[1]

In consequence of the work of Sir Almroth Wright, the method of preventing and treating bacterial diseases by vaccines has been much developed. The general principle is to inject into the individual a suspension of dead bacteria. In certain cases the bacteria are subjected to disintegrating processes before being used, but most frequently the vaccines simply contain killed bacterial cells, and the preparation is comparatively simple.

In the case of organisms such as pyogenic cocci, *B. coli*, etc., the growth from a young sloped agar culture is suspended in normal saline. A uniform suspension is necessary, and if clumps are present these must be disintegrated with a shaking-machine, or deposited by centrifuging. A sample is withdrawn for the enumeration of the organisms (*vide infra*), and the vaccine is then sterilised by heating in a water bath at 57° C. for one to two hours. With certain staphylococci a longer exposure is advisable, and sometimes in such cases a higher temperature must be employed. It is probable that the lower the temperature at which the contained bacteria are killed the more efficient is the resulting vaccine. The success of the sterilisation must be tested by transferring some of the heated vaccine to an agar tube and incubating for forty-eight hours. Appropriate doses are then, with all aseptic precautions, measured by means of sterile graduated pipettes, and placed in small glass bulbs or ampoules drawn out to a capillary tube at one end. It is usual to add sufficient 0·5 per cent. phenol in sterile saline to make the contents of the ampoule up to about 1 c.c. The ampoules when charged are sealed, and for use the sealed end is broken off, the contents are sucked up into a sterile hypodermic needle, and injected fairly deeply into the loose subcutaneous tissue, *e.g.* of the arm. The sterility of vaccines should be controlled by making cultures in fluid media both for aerobic and anaerobic organisms; care must be taken that the antiseptic present is sufficiently diluted, *e.g.* in the case of phenols to less than 0·01 per cent. in the culture medium.[2]

[1] The Therapeutic Substances Act, 1925, prescribes that no person shall manufacture for sale vaccines unless he holds a licence for the purpose. This does not apply where a vaccine is prepared specially for the treatment of an individual patient by his own qualified medical practitioner or, at the latter's request, by another similarly qualified.

[2] The Therapeutic Substances Regulations, specify the tests for sterility which must be made in the case of vaccines manufactured for sale under licence.

Recent investigations on bacterial variation have shown that the immunising properties of vaccines may depend greatly on the state of the culture from which they are prepared. In many instances recently isolated virulent cultures yield the most effective products; this is well exemplified in the case of pneumococci and *B. pestis*. Accordingly, much attention is now being devoted to the methods of selecting cultures which will produce the most solid immunity with a view to conferring protection against the corresponding infections. The special methods adopted in preparing vaccines are described in the chapters on the various diseases.

Methods of counting the Bacteria in Dead Cultures.—In the making of vaccines it is, as indicated above, advisable to know roughly the total number of bacterial cells, whether dead or living, present in a culture, for the dead as well as the living contain the antigens which may stimulate the production of antibodies. A sufficiently accurate enumeration of the bacteria in a vaccine emulsion can usually be made by counting a suitably diluted sample on a hæmacytometer slide. For this purpose a special cover-glass is supplied, which is ground thin in the centre so that an oil immersion lens can be used. But in many cases a dry lens is sufficient, especially if a small quantity of stain, *e.g.* methylene-blue, is added to the diluent. The diluent ought also to contain some antiseptic, especially when the organisms are motile.

Wright's method consists in making a mixture of blood (whose content in red blood corpuscles is known) with the bacterial culture, and comparing the number of bacteria with the number of corpuscles. The observer first estimates the red cells in his blood; a capillary pipette with a rubber teat and with a mark near its capillary extremity is then taken, blood is sucked up to the mark, then an air-bubble, and then an equal volume of the bacterial emulsion diluted according to the empirical estimate the observer forms of its strength. The blood and bacterial emulsion are then thoroughly mixed by being drawn backwards and forwards in the wide part of the pipette, a drop is blown out on to a slide, and a blood film is spread which may be stained by Leishman's method. The bacteria and blood corpuscles are now separately enumerated in a series of fields in different parts of the preparation and the total of each added up. As the number of red corpuscles per c.mm. is known, the number of bacteria can be readily calculated. The results obtained by the various methods may show considerable differences. In the case of certain bacteria, *e.g.* the members of the coli-typhoid and cholera groups, when an emulsion of these is mixed with whole blood, the serum of the latter may have a bacteriolytic or an agglutinating action on the organisms, which interferes with the counting. In such cases direct enumeration, as above described, should be adopted.

Fairly accurate results, as regards number of organisms, may with practice be obtained by taking a standard opacity of emulsion, representing a known number of organisms, and diluting down with saline the emulsion of organisms to be tested till this opacity is

reached. Tubes of the same diameter must, of course, be used. Permanent standards for comparison may be made by preparing suspensions of inorganic substances such as barium sulphate. Brown has elaborated this method.[1]

Inoculation of Animals[2]

The animals generally chosen for inoculation are mice, rats, guinea-pigs, rabbits, and pigeons. Care especially must be taken in drawing conclusions from isolated experiments on rabbits, as they are very liable to suffer from intercurrent infections. It must be remembered that different animal species exhibit marked variations in susceptibility to different infections; therefore in examining material for a particular organism one chooses a susceptible class of animal. On the other hand, in investigating a disease of unknown origin it is essential to inoculate a wide variety of species. The age of the animal also may be an important factor. Animals selected for inoculation should be in good condition, the best evidence of this being that they are gaining in weight; preferably they should have been kept in the laboratory for some days. In the case of the mouse and rat the variety must be carefully noted, as there are differences in susceptibility between the wild and tame varieties, and between the white and brown varieties of the latter. Dogs are, as a rule, not suited for experimental inoculation, and the larger animals are expensive. For certain infections particular species of monkeys are required.

Practically all inoculations are performed by means of a hypodermic syringe. The best variety is that of the "Record" type, furnished with needles of platinum-iridium or stainless steel of various bores. Before use, the syringe should be taken apart, placed in cold water, and sterilised by boiling for five minutes. The materials used for inoculation are cultures, animal exudates, or suspensions of tissues. If the bacteria already exist in a fluid there is no difficulty. The syringe is most conveniently filled out of a shallow conical test-glass, which ought previously to have been covered with filter paper and sterilised. If an inoculation is to be made from organisms growing on the surface of a solid medium, either a little ought

[1] Burroughs Wellcome, London, supply a series of standard opacity tubes for the purpose, along with tables in explanation.

[2] Experiments on animals cannot be performed in Britain without a licence granted by the Home Secretary. According to the nature of the experiments and the species of animals employed various certificates are also required.

to be scraped off and shaken up in sterile broth or gelatin-Locke solution to make an emulsion, or a little sterile fluid is poured on the growth, and the latter rubbed into it with the looped wire.[1] Gross particles which might obstruct the needle may be removed by letting the fluid stand in the conical glass for a few minutes or by centrifuging at a low speed. If a solid organ is used for inoculation, it ought to be first cut into small pieces and rubbed up in a sterile mortar with sterile sand and a little sterile distilled water; if the emulsion is allowed to stand in a conical glass for a few minutes gross particles soon settle to the foot.

The methods of inoculation generally used are: (1) by scarification of the skin; (2) by subcutaneous injection; (3) by intraperitoneal injection; (4) by intravenous injection; (5) by injections into special regions, such as the anterior chamber of the eye, the cardiac chambers, the substance of the testis, brain, etc. Of these (2) and (3) are most frequently used. When an anæsthetic is to be administered, this is conveniently done by placing the animal, along with a piece of cotton wool or sponge soaked in ether, etc., under a bell-jar or inverted glass beaker of suitable size. Another method, *e.g.* as a preliminary to intratesticular inoculation in rabbits, is to inject subcutaneously, three-quarters of an hour beforehand, a dose of 1·5 grams of urethane per kilo. of body weight, and to keep the animal warm.

1. *Scarification.*—Several parallel scratches are made in the skin of the abdomen previously shaved and cleansed, just sufficiently deep to draw blood, and the infective material is rubbed in with a platinum eyelet. The disadvantage of this method is that the inoculation is easily contaminated.

2. *Subcutaneous Injection.*—The hair is cut off the part to be inoculated, and the skin purified with alcohol. The skin is then pinched up, and, the needle of the syringe being inserted, the requisite dose is administered. The wound is sealed with collodion. In the case of mice and tame rats, an assistant holds the animal by the tail and the skin at the nape of the neck.

3. *Intraperitoneal Injection.*—The hair over the lower part of the abdomen is cut, and the skin cleansed. The whole thickness of the abdominal walls is then pinched up between the forefinger and thumb of the left hand, and the needle is passed through the fold thus formed. If the wall is then relaxed, the point of the needle will be within the abdominal cavity, and the inoculation can thus be made. In the case of a mouse the animal is held with the head downward and the left leg stretched out backward, then the needle is introduced near the left groin.

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[1] Suspensions of bacteria in saline should be used without delay, as some organisms, *e.g.* gonococci or pneumococci, may soon die in it.

4. *Intravenous Injection.*—In the rabbit the vein most usually chosen is one of the auricular veins. The part has the hair removed, the skin is cleansed, and the vein made prominent by pressing on it between the point of inoculation and the heart. The needle is then passed obliquely into the vein, and the fluid injected. That it has perforated the vessel will be shown by the escape of a little blood; and that the injection has taken place into the lumen of the vessel will be known by the absence of any swelling in the surrounding subcutaneous tissues. In mice and rats a very fine short needle, as for intracutaneous inoculation, is used to enter the tail vein, which has been previously dilated by heating. In the guinea-pig the jugular vein may be used or a superficial vein which courses on the dorsal and inner aspect of the hind leg (Roth). The latter usually runs diagonally across the leg from the dorsal aspect below to the inner aspect above. In order to expose the vein it is compressed above and a linear incision about 0·25 inch long is made parallel to it through the overlying skin and the subcutaneous tissue is then pushed aside with pointed forceps.

Intracutaneous injection is used to test for a local inflammatory reaction, *e.g.* in order to ascertain the virulence of diphtheria bacilli or to determine sensitiveness to tuberculin. A syringe with a short fine needle (I.S.W.G. 25 or 26) is used; the skin, previously cleansed with gauze soaked in spirit, is pinched into a fold with the thumb and forefinger of the left hand, and the needle, with the eye pointing toward the operator, is passed tangentially into the substance of the skin, entrance into the lax subcutaneous tissue being avoided. An amount of 0·05 to 0·2 c.c. is introduced; a small wheal indicates successful injection. When a series of injections is given, these should be about 1 inch apart. With animals such as the guinea-pig, light-coloured or white specimens are chosen, since the results are more easily read. The areas to be injected (preferably on the abdomen) are first of all clipped and then depilated by applying a fresh 5 per cent. solution of sodium sulphide. After one minute the solution is mopped off, and, if necessary, the treatment is repeated until the area is bare (irritation of the skin will result if the application of the solution is unduly prolonged). Finally, the skin is washed well with water and then with spirit.

Intraspinal injection in the rabbit can be made though the space between the seventh lumbar and first sacral vertebræ; the spine of the former lies in a line with the iliac crests. Other operative procedures in special regions of the body are carried out with the usual aseptic precautions.

Subdural Inoculation.—In the case of rabbits the animal is anæsthetised with ether, and the hair between the eyes is shaved; the bare area is then cleansed. A sagittal incision about 1 inch long is made $\frac{1}{8}$ to $\frac{1}{4}$ inch to one side of the mid-line, with its centre opposite the middle of the eyes. The mesial flap is pulled aside and a small hole sufficient to admit a hypodermic needle is drilled at the point where a line through the middle of the eyes crosses the mid-line of the skull. (A small wedge-shaped, blunt-pointed drill with a cutting edge should be used in order to avoid opening the dura and damaging the brain.) When the dura is exposed a fine needle can be passed horizontally into the subdural space for about $\frac{1}{4}$ inch; 0·2 to 0·4 c.c. may be injected slowly. The skin flaps are sutured, and sealed with collodion. For cleansing the skin

before incising, painting with a mixture of brilliant green and crystal violet, 0·5 gram each, dissolved in 100 c.c. absolute alcohol, is most effective ; the area is ready when the alcohol has evaporated.

Intracerebral injection in the rabbit is carried out as for subdural inoculation (*vide supra*), except that the skull is drilled at a point 2 mm. lateral to the median plane on an imaginary line joining the two outer ocular canthi. A fine hypodermic needle is then passed into the brain to a depth of 3 mm. ; 0·45 c.c. may be injected.

If it is desired to make the injection into the occipital lobe, the hole is drilled at a point 2 mm. lateral to the sagittal suture and 1·5 mm. anterior to the lambdoidal suture.

Intracerebral injection in mice is made as follows : under an anæsthetic the skin over the vertex is depilated and cleansed, and a fine needle is passed for about ⅛ inch through the bone slightly behind and to the side of the vertex ; about 0·05 c.c. may be injected.

After inoculation, the animals ought to be kept in metal cages, which can be thoroughly disinfected subsequently. Preferably they should open from above; otherwise material which may be infective may be scratched out of the cage by the animal.[1] They can be sterilised by boiling in a large fish-kettle or by autoclaving. The general condition of the animal is to be observed, and in any experiment in which the animal survives for some time it should be weighed at regular intervals, *e.g.* weekly. The temperature is usually taken *per rectum*. An ordinary clinical thermometer is smeared with vaseline, and the bulb inserted just within the sphincter, where it is allowed to remain for a minute ; it is then pushed well into the rectum for five minutes. If this precaution be not adopted a reflex contraction of the vessels may take place, which is likely to vitiate the result by giving too low a reading. The normal rectal temperature of the rabbit is on the average 102·4° F., but marked variations occur, and up to 104° F. there is no significant increase. Mice are kept singly in cylindrical glass jars measuring 9 by 5¼ inches. The cover for a jar consists of a rim of tinned iron to which a disc of fine mesh monel metal gauze is soldered ; a lead bar across the top gives sufficient weight.

Collodion Capsules.—These have been used to allow the sojourn of bacteria within the animal body without their coming into contact with the cells of the tissues. Various substances in solution can pass in either direction through the wall by diffusion, but the wall is impermeable alike to bacteria and leucocytes. The following method of preparing such capsules is that of McRae modified by Harris : A gelatin capsule, such as is used by veterinary surgeons, is taken, and in one end there is fixed a small piece of thin glass tubing by gently heating the glass and inserting it. The tube becomes fixed when quite cold, and the junction is then painted

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[1] Miles and Mitchell describe an inexpensive form (*Journ. Path. Bact.* 1934, xxxviii. p. 501).

round with collodion, which is allowed to dry thoroughly. The bore of the tubing is cleared of any obstructing gelatin, and the whole capsule is dipped into a solution of collodion so as to coat it completely. The collodion is allowed to dry, and the coating is repeated; it is also advisable to strengthen the layer by further painting it at the extremity and at the junction. The interior of the capsule is then filled with water by a fine capillary pipette, and the capsule is placed in hot water in order to liquefy the gelatin, which can be removed from the interior by means of the fine pipette. The sac is filled with broth and is placed in a tube of broth It is then sterilised in the autoclave. A small quantity of the broth is removed, and the contents are inoculated with the particular bacterium to be studied, or an emulsion of the bacterium is added. The glass tubing is seized in sterile forceps, and is sealed off in a small flame a short distance above the junction. The closed sac ought then to be placed in a tube of sterile broth to test its impermeability. The result is satisfactory if no growth occurs in the surrounding medium. The sac with its contents can now be transferred to the peritoneal cavity of an animal.

Autopsies on Animals dead or killed after Inoculation.—These should be made as soon as possible after death—in fact, it is preferable to kill the animal when it shows serious signs of illness. It is necessary to use shallow troughs, made of metal or of earthenware. The animal is stretched out and firmly fixed by stout pins to a wooden board which is laid in the trough. The size of the trough will therefore have to vary with the size of the outstretched body of the animal to be examined. Usually the surface of the animal should be soaked in 10 per cent. liquor cresolis saponatus, or in corrosive sublimate (1 : 1000), before it is tied out. This not only to a certain extent disinfects the skin, but, what is more important, prevents hairs which might be infected with pathogenic products from getting into the air of the laboratory. The instruments necessary are scalpels (preferably with metal handles), dissecting forceps, and scissors. They are to be sterilised by boiling for five minutes. Two sets at least ought to be used in an autopsy, and they may be placed, after boiling, on a sterile glass plate covered by a bell-jar. It is also necessary to have a medium-sized hatchet-shaped cautery, or other similar piece of metal. It is well to have sterile capillary pipettes stored in a sterile cylindrical glass vessel, and also some larger sterile glass pipettes. The hair of the abdomen of the animal is removed. If some of the peritoneal fluid is wanted, a band should be cauterised down the linea alba from the sternum to the pubes, and another at right angles to the upper end of this; an incision should be made in the middle of these bands, and the abdominal walls thrown to each side. One or more capillary tubes should then be filled with the fluid

collected in the flanks, the fluid being allowed to run up the tube and the point sealed off ; or a larger quantity may be taken in a sterile pipette. If peritoneal fluid be not wanted, then an incision may be made from the episternum to the pubes, and the thorax and abdomen opened in the usual way. The organs ought to be removed with another set of instruments, and it is convenient to place them pending examination in deep sterile Petri's capsules. It is generally advisable to make cultures and film preparations from the heart's blood. To do this, open the pericardium, sear the front of the right ventricle with a cautery, make an incision in the middle of the part seared, and remove some of the blood with a capillary pipette for future examination ; or, introducing a looped wire, inoculate tubes and make cover-glass preparations at once. To examine any organ proceed as on p.169 . Fix pieces of the organs for microscopic examination (p. 132). The organs ought not to be touched with the fingers. When the examination is concluded, the body should have corrosive sublimate or carbolic acid solution poured over it, and be forthwith burned. The dissecting trough and all the instruments ought to be boiled for half an hour. The amount of precaution to be taken will, of course, depend on the character of the bacterium under investigation, but as a general rule every care should be used.

Methods of obtaining Pathological Material for Examination

All specimens intended for bacteriological examination should be taken with such precautions as will reduce to a minimum the chances of contamination from external sources. At the same time admixture with antiseptics must be avoided. Specimens must be received directly into sterile vessels which are at once closed with suitable sterile stoppers.[1] In most cases the examination (making of cultures, films, animal inoculation) should be carried out soon after withdrawal of the material from the body, as many pathogenic organisms soon tend to die or, when present in mixtures, to be overgrown.

Method of obtaining Blood for Cultivation, Serum, etc.— In the human subject this should be drawn from a vein. The skin of the arm at the bend of the elbow is cleansed with sterile gauze swabs soaked in alcohol or the mixture of brilliant green and crystal violet (p. 163) ; then a tourniquet, *e.g.* several turns of stout bandage

[1] Containers for sputum, etc., to be examined for tubercle bacilli must not be closed with ordinary corks, as these may contain acid-fast organisms.

or indiarubber gas tubing, is applied round the upper arm about the middle of the biceps so as to render the veins turgid, but not to obliterate the radial pulse. Congestion of the veins is aided by the patient opening and closing his hand vigorously; in difficult cases the arm may be immersed in a basin of warm water. (In stout subjects the vein may be palpable to the finger as an elastic cord when it cannot be seen.) The skin at the elbow is rendered tense from behind by the operator's left hand; the syringe, with needle attached, is held in the right hand and almost parallel with the patient's arm; then the needle, with the eye turned away from the skin, is inserted into a prominent vein. The median basilic is usually the vein which is most easily entered, the needle being pointed either in the direction of or against the blood stream. From 5 to 10 c.c. of blood are drawn up into the syringe, the operator's left hand holding the syringe steady while the plunger is withdrawn with the right. The tourniquet is then at once removed, otherwise a hæmatoma tends to form; thereafter the needle is withdrawn from the vein, and the contents of the syringe are at once emptied into the test-tube or flask of culture medium (the mouth of which must be flamed when the plug is removed), which is then plugged again immediately.[1] The syringe and needle must be thoroughly washed out without delay. Another method is to insert a needle into the vein and to allow the blood to flow directly from it into the tube; this is satisfactory for obtaining blood for Wassermann reaction, etc., but is not satisfactory where sterility is essential. The wound may be sealed with gauze and collodion. In making blood cultures in order to detect the presence of enteric bacilli 5 c.c. of blood are added to 10 c.c. of sterilised ox bile or 50 c.c. of a 0·5 per cent. solution of bile salts in 1 per cent. sodium citrate. In examining for other organisms, *e.g.* streptococci, 5 or 10 c.c. of blood are added to a large quantity of fluid medium (100 c.c. or more). Douglas and Colebrook recommend the addition to broth of trypsin, which both prevents clotting of the added blood and also destroys its antibacterial properties. They use a solution [2] of which 1 part is mixed with 20 parts of broth and tubed in amounts of 5 c.c., which are incubated at 37° C. for forty-eight hours, both aerobically and anaerobically, to control sterility. The mixture keeps in the ice-chest for several weeks; 1 c.c. of blood is added to 5 c.c. of trypsin broth. For the cultivation of streptococci from the blood in cases of endocarditis, H. D. Wright recommends the use of broth containing 0·15 to 0·2 per cent. sodium citrate. If it is desired to ascertain the number of living bacteria in the blood, the specimen should be mixed with an equal volume of a solution of sodium citrate 3 grams and sodium chloride 6 grams in 1000 c.c. of water, which is distributed in tubes and sterilised. Coagulation is thus prevented and known quantities of blood can then be incorporated in melted agar and poured into Petri plates.

For obtaining the blood one uses a 10 c.c. syringe of "Record" or "all glass" type provided with a sharp needle, 5 cm. long and 1·2 to 0·85 mm.[3] in external diameter; the separate parts should be

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[1] Bottles fitted with screw-caps (p. 55) may be used as containers for the medium.

[2] Allen & Hanburys' "trypsin for bacteriological purposes."

[3] I.S.W.G.. 18–20.

placed in cold water and sterilised by boiling in plain water for five to ten minutes (they should not be brought into contact with any antiseptic or sodium carbonate). The parts of the syringe before use should be laid on a sterile surface (*e.g.* a sheet of paper which has been passed repeatedly through a Bunsen or spirit lamp flame) and should be similarly covered and then be put together by means of sterile forceps when cool (the needle and the nozzle of the syringe must not be touched with the fingers). The syringe should finally be placed in a sterile container [1] and kept closed until required.

The "Behring venule" [2] is a complete apparatus, supplied sterile, for withdrawing a specimen of blood from a vein. For making blood cultures a form is provided containing suitable media with which the blood automatically mixes.

When only a small sample of blood serum is required the following procedure may be employed. The circulation in the hand is stimulated, *e.g.* by immersion in hot water or by vigorous shaking of the arm. Then the skin of a finger at the base of the nail is swabbed with gauze moistened with spirit. A length of narrow tape is wound round the finger from the base to the terminal phalanx so as to congest the finger tip. Then a puncture through the cleansed skin is made with a sharp triangular needle which has been sterilised by flaming. The blood is collected in a piece of straight quill tube which has been drawn out to capillary ends and which is of such a length that it will fit into the bucket of the centrifuge. The tube is held almost horizontal during the process of filling. If blood ceases to flow a further quantity can be usually obtained without a second puncture by taking off and reapplying the tape. When the tube is two-thirds full the empty part is warmed in a flame and the tip finally sealed. As this end cools the column of blood is retracted into the tube so that the other end can then be sealed. In this way several c.c. of blood may be obtained. Separation of serum is aided by centrifuging the tube.

Cerebro-spinal Fluid—Lumbar Puncture.—This diagnostic procedure, which is often called for in cases of meningitis, can be carried out with a sterilised "antitoxin needle" as follows: The patient should lie on the right side, with knees somewhat drawn up and left shoulder tilted somewhat forward, so that the back is fully exposed. The skin over the lumbar region is then carefully cleansed and sterilised with alcohol and ether, and the hands of the operator should be thoroughly purified. The spines of the lumbar vertebræ having been counted, the left thumb or forefinger is pressed into the space between the third and fourth spines in the middle line; the needle is then inserted about half an inch to the right of the middle line at this level (or the needle may be inserted in the middle line) and pushed through the tissues, its course being directed slightly inwards and upwards, till it enters the subdural space. When this occurs, fluid passes along the needle, sometimes actually spurting out, and should be received in a sterile test-tube. Several cubic centimetres of fluid can thus usually be obtained, no suction being required; where there is no increase of pressure not more

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[1] Metal cases with screw lids to accommodate "Record" syringes are supplied by the Surgical Manufacturing Co., 83–85 Mortimer Street, London, W.1.

[2] Supplied by Bayer Products Ltd., London.

than 5–10 c.c. fluid should be removed.[1] The depth of the subdural space from the surface varies from a little over an inch in children to 3 inches, or even more, in adults—the length of the needle must be suited accordingly. In making the puncture it is convenient to have either a sterile syringe attached, or to have the thick end of the needle covered with a pad of sterile wool, which is, of course, removed at once when the fluid begins to flow. It is advisable to use the platinum-iridium needles which are specially made for the purpose, as a sudden movement of the patient may snap an ordinary steel needle; the needle should be of small bore, as larger needles inflict much pain.[2] The patient must be kept lying down for some hours afterwards.

Fæces.—Specimens intended for bacteriological examination should not be mixed with urine. For the detection of bacilli of the enteric and dysentery groups a loose motion must be obtained, and in the case of suspected carriers it may be necessary to administer a purgative, *e.g.* calomel. On the other hand, for the detection of cysts of *Entamœba histolytica* portions of mucus from the surface of solid motions are selected. Of a loose motion 1 c.c. is sufficient. The usual sterilised swab soaked in the fæces and replaced in its test-tube is satisfactory when there is no delay in making the examination. In other cases the fæces should be placed in a wide-mouthed 2-oz. bottle by means of a small metal spoon which is fitted into the stopper for the purpose, the whole apparatus having been sterilised beforehand by autoclaving. In children especially, a convenient method is to pass a sterile glass speculum into the anus and through this introduce a swab rather stouter than the usual throat swab. When the making of cultures for organisms of the enteric ard dysentery groups must be delayed for more than several hours after the specimen has been taken, it is advisable to add to one volume of the fæces two volumes of 30 per cent. neutral glycerol in 0·6 per cent. NaCl solution and to make a thorough mixture (Teague and Clurman). The presence of the glycerol prevents the suppression of the specific organisms by *B. coli* which would otherwise occur.

Secretions from Throat, etc.—Specimens of these secretions are obtained by rubbing the surface with a "swab" of cotton wool wrapped round the end of a strong wire. The swab is kept in a narrow test-tube of stout glass, and the other end of the wire is fixed in the cork stopper. The whole apparatus is sterilised by dry heat and kept ready for use. In taking specimens from the throat it is important that no antiseptic should have been applied (*e.g.* as a gargle) for some hours before.

Secretion from the Naso-pharynx.—A specimen of pharyngeal mucus may be obtained by means of a swab of cotton wool on the end of a metal wire. The wire ought to be longer than that used in the case of a throat swab and bent near the extremity. A tongue depressor is used; the wire is introduced into the mouth, and passed up behind the soft palate and then brought into contact with the posterior pharyngeal wall. Care must be taken not to touch any

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[1] The fluid should be collected in several tubes, since blood due to the operation, may be mixed with the fluid which escapes at first. Admixture with blood from this source renders the specimen unsuited for examination, except for detecting the presence of organisms.

[2] External diameter about 0·035 to 0·01 inch.

part of the mucous membrane of the mouth. The best method, however, is by means of West's tube. This consists of a glass tube shaped like a catheter, in the interior of which is a thin wire bearing the swab, the latter being just within the end of the tube. The bend of the tube can be used to depress the tongue, or a depressor may be used, and when the tube is sufficiently introduced it is turned up behind the soft palate, and the end of the wire is pressed so as to protrude the swab which is brought into contact with the pharyngeal wall. The swab is then drawn back into the tube (in fact, it usually springs back) and the tube is removed from the mouth. It is important to avoid contamination with mouth organisms, which tend to inhibit the growth of meningococci. Cultures for the latter must be made at once and placed in the incubator as quickly as possible, as cold has an injurious effect on the organisms. If the inoculations have been made at some distance from the laboratory, the plates should be carried in an apparatus with a hot-water jacket—a bag containing a hot-water bottle is often sufficient.

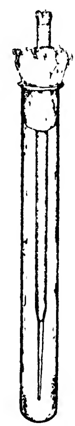


FIG. 25.—Test-tube and pipette arranged for obtaining fluids containing bacteria.

Urine.—In such an examination care must be taken to prevent the contamination of the urine by extraneous organisms. In the male, specimens withdrawn by a sterile catheter into a sterile vessel are preferable, but it is often sufficient to wash thoroughly the glans penis and the meatus with 1 : 1000 corrosive sublimate —the lips of the meatus being everted for more thorough cleansing; the urine is then passed into a series of sterile flasks, the first of which is rejected in case contamination has occurred. In the female, after similar precautions as regards external cleansing, a sterile catheter must be used. If organisms are scanty, the specimen should be centrifuged and films and cultures made from the sediment. Sometimes in examining for tubercle bacilli it may be advisable to collect the twenty-four hours' urine in the ordinary way and to remove some of the sediment which has settled and concentrate the latter in the centrifuge.

Post-mortem Material.—Fluids from the body cavities, pus, urine, etc., may be secured with sterile pipettes (see Fig. 25). The end of the pipette should be plugged with a small piece of cotton wool which is inserted before sterilising ; it prevents the accidental sucking up of infective material.

Solid organs to be examined should, if possible, be obtained whole. They may be treated in one of two ways. (1) The surface over one part at least an inch broad is seared with a cautery heated to dull red heat. All superficial organisms are thus killed. An incision is made in this seared zone with a sterile scalpel, and small quantities of the juice are removed by a platinum spud to make cover-glass preparations and plate or smear cultures (a piece of quill tube drawn out in the flame till slightly narrowed at one end which is then broken across so as to leave a jagged end is an

excellent substitute for the spud). (2) An alternative method is as follows: The surface is sterilised by soaking it well with 1 : 1000 corrosive sublimate for half an hour. It is then dried, and the capsule of the organ is cut through with a sterile knife, the incision being further deepened by tearing. In this way a perfectly uncontaminated surface is obtained.

The clinical history of the case will often suggest what the procedure ought to be in examination. Organisms may occur in the tissues of cadavers apart from disease processes, owing to invasion *ante* or *post mortem*. Among those specially likely to be found are *B. coli*, staphylococci, non-hæmolytic streptococci and *B. welchii*. Burn has shown that the organs most likely to be infected in this way are the lungs and then, in descending order of frequency, kidneys, liver, spleen, and heart blood. In bodies placed at 10° C. soon after death there was no marked difference as regards the presence of bacteria after one hour and forty-eight hours.

CHAPTER V

RELATIONS OF BACTERIA TO DISEASE—INFECTION —PRODUCTION OF TOXINS BY BACTERIA

Introductory.—In most cases, before micro-organisms produce disease they must obtain a foothold in the body, multiply and carry on their vital processes there. These acts of penetration and multiplication are called "infection." The disturbances in structure and function which result in the host in consequence of infection by a parasite constitute the infective disease. An essential feature of such diseases and one which accounts for their very manifold nature is that the etiological factor is living. Accordingly in the interaction between the host and the parasite two living agents are concerned each of which is capable of extensive adaptation. The factors on the part of the host which tend to antagonise the parasite are summed up in "resistance"—the converse being "susceptibility." The capacity of the organism to produce disease is described as "virulence." It has already been stated that a strict division of micro-organisms into true *parasites* and *saprophytes* cannot be made. No doubt there are organisms, such as the tubercle bacillus, gonococcus, etc., which are in natural conditions always parasites associated with disease. But these can lead a saprophytic existence in specially prepared conditions, and there are many of the disease-producing organisms, such as the organisms of typhoid and cholera, which can survive in nature under favourable conditions. A similar statement applies to the terms *pathogenic* and *non-pathogenic*. By the term pathogenic is meant the power which an organism has of producing morbid effects in the animal body, either under natural conditions or in artificial conditions experimentally arranged. Now we know of no organisms which will in all circumstances produce disease in all animals, and, on the other hand, many bacteria described as harmless saprophytes will produce pathological changes if introduced in sufficient quantity. The term pathogenic, therefore, is merely a relative one, and indicates that in certain circumstances the organism will produce

disease, though it is often used for convenience as implying that the organism produces disease in man in *natural* conditions. The term '*commensal*' is applied to organisms normally present as saprophytes on the skin or mucous surfaces of the body. Some commensals, *e.g.* staphylococci and streptococci, have the capacity under certain conditions of invading the tissues and producing pathogenic effects. This is a matter of importance in connection with the origin of natural infections.

It will be shown more fully below that many of the pathogenic bacteria, as regards their morphological and cultural characters, are very similar to organisms normally present in the sites at which the respective lesions are produced. Thus the whole typhoid-dysentery group is related to the *B. coli*, and the meningococcus to various Gram-negative diplococci which abound in the naso-pharynx. This suggests strongly that the specific pathogens have been derived from saprophytic commensals, but the circumstances under which such transformation takes place are unknown, and it has not been effected with certainty under experimental conditions. A few organisms are known which are capable of invading the body and even multiplying extensively in the blood, but which appear to be practically devoid of pathogenic action, *e.g. Spirillum minus* in the mouse and *Trypanosoma lewisi* in the rat. A similar behaviour is shown also in the infection of the rat with the Rickettsia of typhus fever. In the mouse, inoculation with *Tr. pallidum* produces no lesions or other disease effects, but the spirochætes persist for long periods in the body and they invade the central nervous system.

Modifying Conditions.—In studying the pathogenic effects in any instance, the animal affected as well as the micro-organisms must be considered, and not only the species of each, but also its exact condition at the time of infection. In other words, the resulting disease is the product of the sum-total of the characters of the subject of infection, on the one hand, and of the infecting agent, on the other. We may, therefore, state some of the chief circumstances which modify each of these two factors involved, and, consequently, the diseased condition produced.

1. The Subject of Infection.—Here we shall consider only individuals who have not previously been infected with the micro-organism in question, since such earlier contact with the organism or its products may bring about specific changes in reactivity which are dealt with later in the Chapter on Immunity. Among healthy individuals susceptibility to a

particular microbe may vary according to (*a*) species, (*b*) race and individual peculiarities, (*c*) age. Different species of the lower animals show the widest variation in this respect, some being extremely susceptible, others highly resistant, *e.g.* the susceptibility of equines to the bacillus of glanders and the resistance of horned cattle. Then there are parasites, such as that of syphilis, which under natural conditions are peculiar to the human subject and produce disease experimentally in only a few of the lower animals. And again, there are others, such as the gonococcus with which the typical lesions cannot be experimentally reproduced in animals, or appear only imperfectly. although pathogenic effects may follow inoculation with the organisms. The effect of race or "line" on resistance to experimental infections has recently been investigated. Webster and Schütze and others have shown that different lines of mice may vary markedly in their susceptibility to intraperitoneal inoculation with Salmonella bacilli, while manifesting no differences to similar infection with other organisms, *e.g.* Pasteurella bacilli or pneumococci, etc. In the case of the human subject, differences in susceptibility to a certain disease are found amongst different races, and also amongst individuals of the same race, as is well seen in the case of tubercle and other diseases. Age also plays an important part, young subjects being more liable to certain diseases, *e.g.* fungus infections of the hair (ringworm). Where the susceptibility to an infection decreases with age, however, as in the case of diphtheria, there is always the possibility that a part may be played by the specific factor of exposure to the infective agent and the development of immunity through repeated mild infections—"subclinical" infections. On the other hand, at very early ages there may be a relative insusceptibility. For instance, Burky found that young rabbits withstood a larger dose of staphylococcus toxin than mature animals, *e.g.* the mothers. Children under two months old show a similar insensitiveness of their skin to injections of killed staphylococci (Kobak and Pilot). Further, at different periods of life certain parts of the body are more susceptible; for example, in early life, the bones and joints to tuberculous and acute suppurative infections.

In increasing the susceptibility of a given individual, conditions of *general or local diminished vitality* play the most important part. It has been experimentally proved that exposure to cold, fatigue, starvation, etc., all diminish the natural resistance to bacterial infection. Rats naturally immune to glanders can be rendered susceptible by being fed with

phloridzin, which produces a sort of diabetes. Again, the intact skin and mucous membranes act as an efficient protection against the entrance into the deeper tissues of many organisms; in fact, the latter seem often to be actively destroyed on the surface. A local susceptibility may be produced by injuring or diminishing the vitality of a part. If, for example, previous to an intravenous injection of staphylococci, the aortic cusps of a rabbit be injured, the organisms may settle there and set up an ulcerative endocarditis; or if a bone be injured, they may produce suppuration at the part, whereas in ordinary circumstances these lesions would not take place. The work of Findlay has suggested that on injury to the tissues there is liberation locally of histamine-like substances which lead to dilatation of the capillaries along with increased permeability, so that organisms in the circulation will pass out and localise in the area. The local effect of certain tissue extracts in enhancing the response of the skin to various infections and toxins seems to be a similar phenomenon, *e.g.* testicular extract (Duran-Reynals).

In addition to such conditions as have been mentioned, there are also some special factors which predispose to infection. In the case of silicosis of the lungs a marked liability to infection with the tubercle bacillus has long been recognised, and it has been shown by the experimental work of Kettle and Gye that silica has a special effect in making the tissues susceptible to invasion by this organism. Another striking example is the remarkable effect which ionisable calcium salts have in leading to infection with the *B. welchii* and other anaerobes, as was first observed by Bullock and Cramer. They showed that when, for instance, *B. welchii* free of its toxin is injected into a mouse or guinea-pig it rapidly becomes destroyed by lysis or by phagocytosis, whereas if some solution of calcium chloride is injected along with the organism or its spores, a spreading gas gangrene results. A similar effect may follow, though not so readily, when the calcium salt is injected at some part of the body other than the site of injection of the bacilli. In contrast to this, salts of magnesium, sodium, potassium, etc., are devoid of such favouring effects. Calcium salts accordingly seem to have a special action in breaking down the defences of the tissues or leading to a state of "kataphylaxis," as these workers have called it. These results suggest that other specific factors as yet unknown may be concerned in the origin of infections occurring naturally.

Apart from the effect of the state of general nutrition on

susceptibility to infections, a special position is occupied by vitamin A. When animals are fed for a considerable period on food which lacks this accessory factor they tend to develop spontaneous pyogenic infections such as xerophthalmia, abscesses in the mouth, nasal sinuses and middle ear, broncho-pneumonia, enteritis, and infections of the urinary tract: that is, the surface commensals are apparently enabled to invade the tissues. A diet deficient in this vitamin causes alterations in the epithelium lining ducts and gland acini, which becomes keratinised; and it is suggested that the consequent obstruction of ducts by masses of these desquamated cells offers a favourable nidus for bacterial growth and so is responsible for the infection. Lassen has shown, however, that there is also a general diminution in resistance, since rats which have developed signs of vitamin A deficiency are more susceptible to subcutaneous or intravenous inoculations of paratyphoid bacilli than are normal or under-nourished animals. The change is attributed to functional impairment of the reticulo-endothelial system. According to Green and Mellanby, the addition of carotene to diets lacking vitamin A restores the anti-infective property. Harde and others have found that ascorbic acid protects guinea-pigs against an otherwise fatal dose of diphtheria toxin.

Such facts, established by experiment (and many others might be given), illustrate the important part which local or general conditions of diminished vitality may play in the production of disease in the human subject. This has long been known by clinical observation. In normal conditions the blood and tissues of the body, with the exception of the skin and certain of the mucous surfaces, are bacterium-free, and if a few organisms gain entrance, they are destroyed. But if the vitality becomes lowered, their entrance becomes easier and the possibility of their multiplying and producing disease greatly increased. In this way the favouring part played by fatigue, cold, etc., in the production of diseases of which the direct cause is a bacterium may be understood. It is important to keep in view in this connection that many of the inflammation-producing and pyogenic organisms are normally present on the skin and various mucous surfaces; and also that during epidemics of a disease, *e.g.* typhoid, cholera, meningitis, diphtheria, the pathogenic organisms may be present on the mucous membranes of healthy individuals—that is, may have gained access to the body without producing the disease. The action of a certain organism may devitalise the tissues to such an extent as to pave the way for the entrance of other bacteria; we may men-

tion the liability to the occurrence of pneumonia, erysipelas, and various suppurative conditions, in the course of or following infective fevers. In some cases the specific organism may produce lesions through which the other organisms gain entrance, *e.g.* in typhoid, diphtheria, etc. A notable example of diminished resistance to bacterial infection is seen in the case of diabetes; tuberculosis and infection with pyogenic organisms are prone to occur in this disease, and are apt to be of a severe character. It is not uncommon to find in the bodies of those who have died from chronic wasting disease, collections of micrococci or bacilli in the capillaries of various organs, which have entered in the later hours of life—that is to say, the bacterium-free condition of the blood has been lost in the period of prostration preceding death.

In the course of infections there is a tendency for the body to alter in its behaviour toward the micro-organisms and their products. This altered reactivity or *allergy* manifests itself in various ways and frequently takes the form of super-sensitiveness, *i.e.* intensification of the phenomena of damage or reaction. The view has been advanced that in those infective diseases characterised by a long incubation period, part of this time is occupied by the development of allergy, whereupon fever and the other symptoms appear. In some infections which pursue a chronic or relapsing course, such as syphilis, the early lesions differ markedly in character from those which occur later, the former being relatively mild, the latter severe as regards destructive effects; the features of the late lesions depend on allergy of the tissues. Again, in tuberculosis the difference in the changes in the lung at different ages has been similarly explained. The subject is one of great complexity and it is impossible to say at present how far the usual manifestations of infective disease are allergic in nature. Also the distinction between allergy leading to harmful effects and aggravation of disease, on the one hand, and the opposite, *i.e.* acquired immunity, is not always clearly defined. But it is definitely established that after recovery from an infection the tissues will on a later occasion react more rapidly and more intensely to contact with the same micro-organism (or its products) and that this may result in preventing the latter from again obtaining a foothold.

The methods by which the natural resistance may be specifically increased belong to the subject of *immunity*, and are described in the chapter on that subject.

Carriers.—As has been stated, many of the organisms which

produce inflammatory and suppurative affections are normally present on the skin or mucous membranes ; but it has also been established that several of the causal agents of acute infectious diseases, such as typhoid, epidemic meningitis, diphtheria, etc., may flourish on the mucous membranes of individuals in apparent health. Such individuals are known as "carriers," and they play a highly important part in the spread of these infections. One group of carriers is constituted by those who have suffered from the disease, and in whom the organism persists after recovery, and these are usually designated "temporary" or "chronic" according to the duration of the condition. The proportion of those who become carriers, and the period of carrying the organisms, vary in different diseases. In cholera, for example, the period is usually comparatively short, whereas in typhoid the organisms not infrequently persist for an indefinite period of time. Also, especially in a number of protozoal infections, *e.g.* piroplasmosis, after recovery from the acute attack the organisms persist permanently in the blood ; this state has been termed non-sterilising immunity or "premunition" (Sergent and Parrot). The other group of carriers comprises apparently healthy individuals who harbour the organisms and are not known to have suffered from the disease. Some of these may really have had a mild attack, but in others there is no evidence of this. A few subsequently develop the disease —"precocious" carriers, although the carrier state may occur temporarily without a clinical attack of the disease developing. In many instances previous contact with a case of the disease can be traced. On the other hand, no connection of this kind may be discoverable ; for instance, the meningococcus is often found, especially during epidemics, in "non-contacts," the organism apparently spreading widely from individual to individual in the community. This illustrates a very important principle regarding the spread of infective diseases. The causal organism may be transmitted through several insusceptible individuals in whom it persists or even multiplies for a time without causing recognisable illness, but such persons are capable of infecting a susceptible individual and setting up a typical attack. This explains the failure to establish continuity between successive cases which is often a feature of epidemics. Frequently chronic carriers exhibit some local abnormality, either anatomical in character or of the nature of infection with other bacteria, which favours the persistence of the specific organisms. Further facts will be given in connection with the special diseases.

2. The Infecting Agent.—In the case of a particular species of bacterium, its effect will depend chiefly upon (*a*) its virulence and (*b*) the number introduced into the body. To these may be added (*c*) the path of infection.

The *virulence, i.e.* the power of invading the body, multiplying and producing disease, varies greatly in different conditions, and the methods by which it can be diminished or increased will be afterwards described (*vide* Chapter VI.). Natural barriers which are effective against organisms of low virulence, *e.g.* the skin and mucous membrances, may be penetrated, with the setting up of infection, by the same species when highly virulent. One important point is that when a bacterium has been enabled to invade and multiply in the tissues of an animal, its virulence for that species is often increased. The virulence sometimes may be still more increased by inoculating from one animal to another in series—the method of *passage.* Widely different effects are, of course, produced on the virulence being altered. For example, a streptococcus which produces merely a local inflammation or suppuration, may produce a rapidly fatal septicæmia when its virulence is raised. Virulence also has a relation to the animal employed, as occasionally on being increased for one species of animal it is diminished for another. For example, streptococci, on being inoculated in series through a number of mice, acquire increased virulence for these animals, but become less virulent for rabbits (Knorr). Certain facts suggest that there may be a periodicity in virulence, *i.e.* that an organism may for a time produce a relatively mild type of disease and then develop into a more potent strain capable of overcoming the resistance of a greater number of individuals ; this would account for the fact that in the case of some diseases widespread epidemics occur at almost fixed intervals of years. Virulence is discussed in more detail later (p. 204); but it may be said here that under natural conditions there are two important properties of an organism upon which its virulence depends, (1) *infectivity, i.e.* capacity to flourish in the body of the host and (2) *pathogenicity* or power of causing damage to the tissues.

The *number* of the organisms introduced, *i.e.* the dose of the infecting agent, is another point of importance. The healthy tissues can usually resist a certain number of pathogenic organisms of given virulence. It is only in a few instances that one or two organisms introduced will produce a fatal disease, *e.g.* the case of anthrax in white mice. The healthy peritoneum of a rabbit can resist and destroy a considerable number of

pyogenic micrococci without any serious result, but if a larger dose be introduced, a fatal peritonitis may follow. There is, therefore, for a particular animal, a minimum lethal dose which can be determined by experiment only—a dose, moreover, which is modified by various circumstances difficult to control.

The path of infection may alter the result, serious effects often following entrance into the blood stream. Staphylococci injected subcutaneously in a rabbit may produce only a local abscess, while on intravenous injection multiple abscesses in certain organs may result and death may follow. Again, with a particular species of host, *e.g.* the mouse, one strain of streptococcus may be highly virulent when introduced into the nasal cavity, but not when injected into the subcutaneous tissue, whereas with most virulent strains the opposite is the case (Loewenthal). Local inflammatory reaction with subsequent destruction of the organisms may take place entirely at the site of infection or may occur also in the related lymphatic glands. The latter therefore act as a second barrier of defence, or as a filtering mechanism which aids in protecting against blood infection. This is well illustrated in the case of septic infections of wounds. In some other conditions, however, the organisms are very rapidly destroyed in the blood stream ; thus Klemperer found that, in the dog, subcutaneous injection of the pneumococcus produces death more readily than intravenous injection. The route by which bacteria enter the body under natural conditions varies according to the particular organism, as appears in the different diseases. Certain organisms are highly selective in their affinities for particular tissues or organs. Thus the site of primary infection by the gonococcus is restricted to the genito-urinary tract or the conjunctiva, while the pneumococcus most commonly infects the respiratory tract. On the other hand, many organisms such as the tubercle bacillus and the pyogenic cocci may attack any part. It is these affinities which determine the infectivity of secretions and excretions of affected individuals, a knowledge of which therefore is important for preventing spread of such diseases. Of course, we are ignorant as to the exact factors which are responsible for an organism flourishing, say, on one mucous membrane and not on another. It is important to note that certain pathogenic bacteria can penetrate the intact skin, *e.g.* *B. pestis* in the guinea-pig. Invasion of the tissues may occur rapidly, as is seen in the case of *Tr. pallidum,* which, after being applied to scratches on the scrotal skin of the guinea-pig, has been found in the popliteal lymph glands five minutes later.

Mixed infection with several species of organisms is sometimes of importance in the production of disease. One of the most striking examples of this is swine influenza, which is due to infection with a bacillus of the hæmophilic group together with a filterable virus. Neither of these agents by itself is highly pathogenic, whereas the two together produce a severe condition. More commonly a second organism becomes superadded to a pre-existing infection, especially when a free surface is involved, and so aggravates the primary disease, *e.g.* infection with pyogenic organisms additional to tuberculosis in the lungs where there is cavity formation.

Modes of Bacterial Action.—In the production of disease by micro-organisms there are two main factors involved, namely, (*a*) the multiplication of the living organisms after they have entered the body, and (*b*) the production by them of poisons which may act both upon the tissues around and upon the body generally. The former corresponds to *infection*, the latter is of the nature of *intoxication* or poisoning. In different diseases one of these is usually the more prominent feature, but both are always more or less concerned.

1. *Infection and Distribution of the Bacteria in the Body.*—After pathogenic bacteria have invaded the tissues, or in other words, after infection by bacteria has taken place, their further behaviour varies greatly in different cases. In the human subject after infection one of two things usually happens. Either the organisms remain local, in which case they may produce little reaction around them, as in tetanus, or a well-marked lesion, as in diphtheria, etc. Or on the other hand, they may pass by the lymph or blood stream to other parts or organs in which they settle, multiply, and produce lesions, as in tubercle. In certain cases they may reach and multiply in the blood stream, producing a fatal septicæmia. In the lower animals this multiplication of the organisms in the blood throughout the body may be very extensive (for example, the septicæmia produced by the pneumococcus in rabbits); but in septicæmia in man it is seen in less degree, the organisms rarely appear in large numbers in the circulating blood, and their detection in it during life by microscopic examination is rare, and even culture methods may give negative results unless a considerable amount of blood is used. But in such cases the organisms may be found *post mortem* lying in large numbers within the capillaries of various organs, *e.g.* in cases of septicæmia produced by streptococci. However, in infections with certain spirochætes (relapsing fever) and protozoa

(malaria, etc.) the causal organisms occur in large numbers in the peripheral blood. It is important to draw a distinction between the mere presence of organisms in the blood—*bacteriæmia*, and their active multiplication in it—*septicæmia.* The former condition, which is frequent in many infections, represents merely an overflow of the organisms from the lesions, as is well exemplified in the early stages of typhoid fever. It is also a common occurrence in subacute infective endocarditis where organisms may be cultivated from the blood over a considerable period. But there is no progressive multiplication in such cases, and we may say that in bacteriæmia the organisms would soon disappear if the source of supply were removed. Where a chronic focus of infection exists, however, the continued dissemination of organisms and their products may cause serious effects either upon the body generally or on particular tissues.

2. *Production of Chemical Poisons.*—In all these cases the growth of the organisms is accompanied by the formation of *chemical products,* which act generally or locally in varying degree as toxic substances. The toxic substances become diffused throughout the system, and their effects are manifested chiefly by symptoms such as the occurrence of fever, disturbances of the circulatory, respiratory, and nervous systems, etc. In some cases corresponding changes in the tissues are found, for example, the changes in the nervous system in diphtheria, to be afterwards described. The general toxic effects may be so slight as to be of no importance, as in the case of a local suppuration; or they may be very intense, as in pyæmia; or, again, less severe but producing cachexia by their long continuance, as in tuberculosis.

The occurrence of *local tissue changes or lesions* produced in the neighbourhood of the bacteria, as already mentioned, is one of the most striking results of bacterial action. These are due to chemical substances formed directly by the bacteria or to ferments, etc., liberated from the damaged tissues. In this case it is more difficult to demonstrate the mode of action, for in the tissues the chemical products are formed by the bacteria slowly, continuously, and in a certain degree of concentration, and these conditions cannot be exactly reproduced by experiment. Further, it is very doubtful whether all the chemical substances formed by a certain bacterium growing in the tissues are also formed by it in cultures outside the body (*vide* p. 192). The separated toxin of diphtheria, like various vegetable and animal toxins, however, possesses a local toxic action of very

intense character, evidenced often by extensive necrotic change. It is also to be noted that more than one poison may be produced by a given bacterium, *e.g.* the staphylococcus or tetanus bacillus (pp. 281, 634).

The injection of large quantities of many different pathogenic organisms in the *dead* condition results in the production of a local inflammatory change which may be followed by suppuration, this effect being possibly brought about by certain substances in the bacterial protoplasm common to various species, or at least possessing a common physiological action (Buchner and others). When dead tubercle bacilli, however, are introduced into the blood stream, nodules result in certain parts, which have a resemblance to ordinary tubercles. In this case the bodies of the bacilli evidently contain a highly resistant and slowly acting substance which gradually diffuses around and produces effects (*vide* Tuberculosis).

Summary.—We may say, then, that the action of bacteria as disease-producers depends upon the chemical products formed directly or indirectly by them. This action is shown by *tissue changes* produced in the vicinity of the bacteria or throughout the system, and by *toxic symptoms* of great variety of degree and character. The degree and nature of these changes and symptoms may differ according as the host has or has not come into contact with the microbes previously, *i.e.* according to the presence or absence of allergy.

We shall first consider the effects of bacteria on the body generally, and afterwards the nature of the chemical products.

Effects of Bacterial Action

These may be for convenience arranged in a tabular form as follows:

A. Tissue Changes.

(1) Local changes, *i.e.* changes produced in the neighbourhood of the bacteria.

Position: (*a*) At primary lesion.
(*b*) At secondary foci.

Character: (*a*) Tissue reactions } Acute or
(*b*) Degeneration and necrosis } chronic.

(2) Changes produced at a distance from the bacteria, directly or indirectly, by the absorption of toxins.

(*a*) In special tissues—
  (α) as the result of damage, *e.g.* nerve cells and fibres, secreting cells, vessel walls :
  (β) changes of a reactive nature in the blood-forming tissues and organs.
(*b*) General anatomical changes, the effects of malnutrition or of increased waste.

B. Symptoms and Changes in Metabolism.

The occurrence of fever, of errors of assimilation and elimination, etc.

A. Tissue Changes produced by Bacteria.—The effects of bacterial action are so various as to include almost all known pathological changes. However varied in character, they may be classified under two main headings : (*a*) those of a degenerative or necrotic nature, the direct result of damage ; and (*b*) those of reactive nature, defensive or reparative. The former are the expression of the essential vulnerability of the tissues, the latter of protective powers evolved for the benefit of the host. In the means of defence both leucocytes and certain cells of the tissues are concerned. Both show phagocytic properties, *i.e.* have the power of taking up bacteria into their protoplasm. The cells are attracted to the focus of infection by chemotaxis, and thus we find that different bacteria may attract different cells. The most rapid and abundant supply of phagocytes is seen in the case of suppurative conditions where the polymorphonuclear (neutrophile) leucocytes of the blood are chiefly concerned. When the local lesion is of some extent there is usually an increase of these cells in the blood—a neutrophile leucocytosis. And further, observation has shown that associated with this there is in the bone-marrow an increased number of the mother-cells of these leucocytes—the neutrophile myelocytes. The facts abundantly show that the means of defence is not a mere local mechanism, but that increased proliferative activity in distant tissues is called into play.

In other cases the cells chiefly involved are the mononuclear hyaline leucocytes, and with them the endothelial cells, adventitial cells, etc. ; these form together a group of non-granular cells to which Metchnikoff gave the name *macrophages*, and which more recently have been designated the *reticulo-endothelial system.* These cells are all characterised by their capacity to ingest minute particles, such as bacteria or finely divided suspensions of carbon, iron oxide, etc., and they also store in

granular form certain colloids presented to them in solution, such as the "vital" stains. The cells which possess this phagocytic property are especially the endothelia lining lymph gland sinusoids and the blood sinuses in the spleen, liver, and bone marrow, also the reticulum cells of lymphatic tissue, spleen, and bone marrow, the histiocytes of connective tissue in general, and a proportion of the monocytes in the circulating blood; less active in this respect are the endothelia of other blood and lymph vessels and the fibrocytes. When bacteria such as pneumococci are injected into the blood stream, they are rapidly taken up by the phagocytes, the subsequent events depending on the virulence of the organisms. If they are weakly virulent, they may permanently disappear from the circulation, being eventually destroyed; but in the case of a virulent strain they reappear again in the blood and, in the severest form of infection, progressively increase till death occurs from septicæmia. It is clear that the phagocytes play an important part in the fixation and destruction of bacteria; also, on the analogy of their behaviour toward foreign colloids, it is thought that they may fix circulating toxins similarly. On the other hand, it has not been possible with certainty to produce marked increase in the susceptibility to organisms which cause general infections, by exclusion of a considerable part of the reticulo-endothelial system. Such attempts have taken the form either of splenectomy or of "blockade" by repeated massive injections of substances which are actively stored by the phagocytic cells, or both procedures have been combined. Except in certain infections which are greatly increased in severity after splenectomy, such as that with the spirochæte of relapsing fever, these procedures have not led to definite lowering of resistance. One point which must be borne in mind here is that the proliferative capacity of the cells of the reticulo-endothelial system is so great that the chances of effecting extensive blockade are remote. On the other hand, it has been demonstrated by Ledingham that a local increase of such cells may enhance resistance; thus an area of skin in which the phagocytic cells were present in excess owing to previous infiltration with India ink, failed to develop the usual lesions on the intracutaneous injection of *vaccinia virus* at this site. Similarly, the resistance of serous sacs to infection can be intensified by procedures which lead to a local accumulation of macrophages. Reaction by such cells is well seen in typhoid fever, where the specific bacillus appears to have little or no action on the neutrophile leucocytes. In other cases, again, the reaction may be mainly

on the part of the connective tissue cells, though their proliferation is always associated with some variety of leucocytic infiltration and usually also with the formation of new blood vessels. Such a connective tissue reaction occurs especially in slow infections or in the later stages of an acute infection. The reactive tissue changes in the presence of bacterial invasion are naturally very varied,—examples of this will be found in subsequent chapters,—but they may be said to be manifestations of the two fundamental processes of (*a*) increased functional activity—movement, phagocytosis, secretion, etc.—and (*b*) increased formative activity—cell growth and division. The exudation from the blood vessels has been variously interpreted. There is no doubt that the exudate may have bactericidal or opsonic properties and also acts as a diluting agent, but it must still be held as uncertain whether the process of exudation ought to be regarded as primarily defensive or as the direct result of damage to the endothelium of the vessels. It may also be pointed out that the various changes referred to are none of them peculiar to bacterial invasion; they are examples of the general laws of tissue change under abnormal conditions, and they can all be produced by chemical substances in solution or in a particulate state. What constitutes their special feature is their progressive or spreading nature, due to the bacterial multiplication.

(1) Local Lesions.—In some diseases the lesion has a special site—for example, the lesion of typhoid fever, and, to a less extent, that of diphtheria. In other cases it depends entirely upon the point of entrance, *e.g.* malignant pustule and wound infections due to pyogenic bacteria. In others again, there is a special tendency for certain parts to be affected, as the upper parts of the lungs in tubercle. In some cases the site has a mechanical explanation.

When organisms gain an entrance to the blood from a primary lesion, the organs specially liable to be affected vary greatly in different diseases. Pyogenic cocci show a special tendency to settle in the capillaries of the kidneys and produce miliary abscesses, whilst these lesions rarely occur in the spleen. On the other hand, the nodules in disseminated tubercle or glanders are much more numerous in the spleen than in the kidneys, which in the latter disease are usually free. Thus the distribution of lesions cannot be explained always on a mechanical basis.

The question also arises as to whether particular strains of organisms may have a selective affinity for certain organs or tissues. According to Rosenow, for example, cultures of

streptococci isolated from gall-bladder disease in man when injected intravenously into animals such as rabbits, tend to localise specially in the gall-bladder. General confirmation of this view is lacking, but it appears that sometimes such may be the case. Thus, Helmholz among many cultures of *B. coli* found several which when injected intravenously into rabbits produced chiefly renal lesions.

Acute Local Lesions.—The local inflammatory reaction presents different characters in different conditions. It may be accompanied by abundant fibrinous exudation, by great catarrh (in the case of an epithelial surface), by hæmorrhage, or by œdema ; it may be localised or spreading in character ; it may be followed by suppuration, and may lead up to necrosis of the tissues of the part, a good example of the latter event being a furuncle. Among the pyogenic organisms staphylococci and streptococci habitually show a different behaviour on inoculation into the same species of animal. Thus staphylococci tend to produce large localised suppurations, while the hæmolytic streptococci cause diffuse spreading purulent inflammations. In order to elucidate this difference, Menkin injected a vital dye, trypan-blue, into infected areas of skin in rabbits ; he found that with the staphylococcus there was blocking of the lymphatics of the part within an hour, which was apparently due to its action in causing tissue necrosis. After inoculation with the streptococcus, however, the lymphatics remained permeable for thirty hours. Other examples will be given in subsequent chapters, but it will be seen how complex may be the factors on which the production of a characteristic lesion depends. The necrotic or degenerative changes affecting especially the more highly developed elements of tissues are chiefly produced by the direct action of the bacterial poisons, though aided by the disturbances of nutrition involved in the vascular phenomena. It may here be pointed out that a well-marked inflammatory reaction is often found in animals which occupy a medium position in the scale of susceptibility ; and also that an organism which typically causes a general infection in a certain animal, may produce only a local inflammation when its virulence is lessened.

A characteristic appearance found in many virus infections is the inclusion body, which may occur in the protoplasm of cells or in the nucleus, or in both situations according to the particular disease. Such inclusions, in certain cases at least, represent a change occurring round a colony of virus particles.

Chronic Local Lesions.—In a considerable number of diseases

produced by bacteria the local tissue reaction is a more chronic process than that described; there is less vascular disturbance and a greater preponderance of the proliferative processes, leading to new formation of granulation tissue. This formation may occur in foci here and there, so that nodules result, or it may be more diffuse. Such changes especially occur in the diseases often known as the *infective granulomata*, of which tubercle, syphilis, leprosy, glanders, actinomycosis, etc., are examples.

Neoplasms.—Rous originally showed that a sarcoma in fowls is due to a filterable virus and since then a number of infective tumours in birds have been described. In mammals of several species papillomata are caused by filterable viruses and recently it has been found by Rous that an infective papilloma of cotton-tail rabbits, studied by Shope, when transferred to tame rabbits causes lesions which ultimately become the seat of epithelioma. So far, however, it has not been demonstrated that a virus is essential to the continued growth of this malignant tumour.

(2) General Lesions produced by Toxins.—In the various infective conditions produced by bacteria, changes commonly occur in certain organs unassociated with the actual presence of the organisms; these are due to the action of bacterial products circulating in the blood. Many such lesions can be produced experimentally. The secreting cells of various organs, especially the kidney and liver, are specially liable to change of this kind. Cloudy swelling, which may be followed by fatty change or by actual necrosis with granular disintegration, is common. Hyaline change in the walls of arterioles may occur, and in certain chronic conditions amyloid change is brought about in a similar manner. The latter has been produced in animals by repeated injections of *Staphylococcus aureus*. Capillary hæmorrhages are not uncommon, and are in many cases due to an increased permeability of the vessel walls, aided by changes in the blood plasma, as indicated sometimes by diminished coagulability. Similar hæmorrhages may follow the injection of some bacterial toxins, *e.g.* of diphtheria, and also of vegetable poisons, *e.g.* ricin and abrin. Skin eruptions occurring in the exanthemata are probably produced in the same way; moreover, there is the important fact that similar skin eruptions may be produced by poisoning with certain drugs. (Certain eruptions, however, may be allergic in origin.) In the nervous system degenerative changes have been found in diphtheria, both in the spinal cord and in the peripheral nerves, and have been reproduced experimentally by the products of the diphtheria bacillus. It is probable that some

of the lesions of the nervous system occurring in syphilis have likewise a toxic origin.

B. Disturbances of Metabolism, etc.—It will easily be realised that such profound tissue changes as have been detailed cannot occur without great interference with the normal bodily metabolism. General malnutrition and cachexia are of common occurrence, and it is a striking fact found by experiment that after injection of bacterial products, *e.g.* of the diphtheria bacillus, a marked loss of body weight often occurs which may be progressive, leading to the death of the animal. In bacterial disease assimilation is often imperfect, for the digestive glands are affected, it may be by actual poisoning by bacterial products, it may be by the occurrence of fever, and excretion is interfered with by the damage done to the excretory cells. But of all the changes in metabolism the most difficult to understand is the occurrence of that interference with the heat-regulating mechanism which results in fever. The degree and course of the latter vary, sometimes conforming to a more or less definite type, when the bacilli are selective in their field of operation, as in lobar pneumonia or typhoid, sometimes being of a very irregular kind, especially when the bacteria from time to time invade fresh areas of the body, as in pyæmic affections. The main point of interest regarding the development of fever is as to whether it is a direct effect of the circulation of bacterial toxins, or should be looked on as part of the reaction of the body against the irritant. This question has still to be settled, and all that we can do is to adduce certain facts bearing on it. Thus in diphtheria and tetanus, where toxic action leading to degeneration plays such an important part, fever may be a very subsidiary feature, except in the terminal stage of the latter disease; and in fact in diphtheria profoundly toxic effects may be produced with little or no interference with heat regulation. On the other hand, in bacterial diseases where defensive and reparative processes predominate, fever is rarely absent, and it is nearly always present when there is an active leucocytosis going on. In this connection it may be remarked that several observers have found that when a relatively small amount of the dead bodies of certain bacteria are injected into an animal, fever occurs; while the injection of a large amount of the same is followed by subnormal temperatures and rapidly fatal collapse. Accordingly, it might appear as if the occurrence of fever had a beneficial effect. But such an effect is seldom, if ever, due to the bacteria being unable to multiply at the higher degrees of temperature occurring in fever. A certain amount

of evidence has been brought forward to show that antibodies are more rapidly produced when the temperature of the body is above the normal, and it has been supposed that in this way fever may be of the nature of a defensive reaction. But the production of antagonistic substances may be effected without the occurrence of fever or of any apparent disturbance of health.

Symptoms.—Many of the symptoms occurring in bacterial infections are produced by the histological changes mentioned, as can be readily understood; whilst in the case of others, corresponding changes have not yet been discovered. Of the latter, those associated with fever, with its disturbances of metabolism and manifold affections of the various systems, are the most important. The nervous system is especially liable to be affected—convulsions, coma, paralysis, etc., being common. The symptoms due to disturbance or abolition of the functions of secretory glands also constitute an important group, forming, as they do, a striking analogy to what is found in the action of various drugs.

These tissue changes and symptoms are given only as illustrative examples, and the list might easily be greatly amplified. The important fact, however, is that *nearly all, if not quite all, the changes found throughout the organs (without the actual presence of bacteria), and also the symptoms occurring in infective diseases, either can be experimentally reproduced by the injection of bacterial poisons or have an analogy in the action of drugs.*

The Toxins produced by Bacteria

Early Work on Toxins.—The introduction of the principle of rendering fluid cultures bacteria-free by filtration through unglazed porcelain, and its application by Roux and Yersin to obtain, in the case of the *B. diphtheriæ*, a solution containing a toxin which reproduced the symptoms of this disease (*vide* pp. 373, 384), constitute the starting-point of modern work on the subject. Brieger had previously isolated from putrefying substances, and also from bacterial cultures, nitrogen-containing bases, *ptomaines*, similar to those occurring in the ordinary metabolic processes of the body. Ptomaines isolated from pathogenic bacteria in no case reproduced the symptoms of the corresponding disease, and they have only historic interest.

General Facts regarding Bacterial Toxins.—In dealing with the action of toxins it is necessary to distinguish between the effects produced by the actual constituents of the bacterial

protoplasm (intracellular toxins, endotoxins) and those which are traceable to soluble substances in the media in which bacteria are growing (extracellular toxins, exotoxins). The former are concerned in the action of by far the greater number of pathogenic bacteria; the latter account for the pathogenic processes seen in a limited number of diseases, of which diphtheria and tetanus are the most important. This distinction is important practically, as in consequence of these last two diseases having specially been investigated early in the history of research on the subject, there has been a tendency to take for granted that poisons of a similar constitution are concerned in all cases of bacterial intoxication. However, no definite line can be drawn between the two kinds of toxins, since wide variations occur in the readiness with which different organisms liberate toxic products from their bodies. It is a point of fundamental importance that substances with all the properties of toxins have only been formed by the activity of living organisms.

Exotoxins.—In the cases of a few pathogenic bacteria the media in which they are growing become extremely toxic. The best examples are the diphtheria, tetanus, and botulism bacilli; in these and similar cases when broth cultures are filtered bacterium-free toxic fluids are obtained, which on injection into animals reproduce the characteristic symptoms of the corresponding diseases. This contrasts with such cases as those of the pneumococcus or of *B. anthracis*, filtered cultures of which are usually non-toxic. Poisons appearing in culture media have been called extracellular toxins or *exotoxins*. It is uncertain whether they are excreted by the bacteria or whether they are produced by the bacteria acting on the constituents of the media. The exotoxins are easily obtainable, but no method has been discovered of obtaining them in a pure form. Our knowledge of their properties is chiefly derived from the study of the toxic filtrates of broth cultures—these filtrates being usually referred to as simply as the "toxins." These toxins differ in their effects from the endotoxins in that specific actions on certain tissues are often manifested. Thus the toxins of the diphtheria, the tetanus, and the botulism bacilli all act on the nervous system. With some of the pyogenic bacteria, again, poisons, probably of similar nature, produce lysis of red blood corpuscles, which may in certain instances explain the anæmia so common in the associated diseases. In the action of many of these toxins the occurrence of a period of incubation between the introduction of the poison into the

animals' tissues and the appearance of symptoms is often a marked feature. Exotoxins when injected into living animals act as antigens and lead to the appearance in the blood serum of specific antibodies; these are substances which combine with the corresponding toxins and so neutralise their pathogenic action.

Among the properties of the exotoxins are the following. They are apparently all uncrystallisable; they are soluble in water and they are little dialysable, though they vary in this respect. They are precipitated along with proteins by various salts, and in the case of certain of these, *e.g.* ammonium sulphate, the toxic properties are unaltered when the precipitate is re-dissolved; in fact, toxins may be purified and concentrated to a considerable extent by such means. Abt, by treatment of crude diphtheria toxin with calcium phosphate, has removed 97 per cent. of protein impurity. They are often relatively unstable, having their toxicity diminished or destroyed by heat (the degree of heat which is destructive varies much in different cases and according to the reaction, etc.), light, and by certain chemical agents, especially those which denaturate proteins. Some chemical agents appear to destroy toxin completely, whereas others, *e.g.* formaldehyde, convert it into a non-toxic form which still retains the property of developing antibodies when injected into animals. To the latter modification the name of *toxoid* has been given. Weak acid, *e.g.* acetic, deprives toxin of its toxicity, but the latter is more or less restored on neutralising. In the case of all toxins the fatal dose for an animal varies with the species, body-weight, age, and previous conditions as to food, temperature, etc. Some species of animals show comparatively great constancy of susceptibility to a given toxin; in others individual variations are a marked feature. In estimating the minimal lethal dose of a toxin these factors must be carefully considered.

Endotoxins.—The dead bodies of certain bacteria have been found to have toxic properties. When, for instance, tubercle bacilli are killed by heat and injected into the body tissues of a susceptible animal, tuberculous nodules are found to develop round the sites where they have lodged. From this it is inferred that they must have contained characteristic toxins, seeing that characteristic lesions result. The bodies of such organisms as the pyogenic cocci, *B. typhosus*, the meningococcus, and *V. choleræ*, likewise give rise to pathogenic effects. Toxins occurring in the fluids in which such bacteria are living are chiefly the result of the disintegration of the organisms, which is

always occurring in any bacterial growth. This is well seen in the case of Shiga's dysentery bacillus; filtrates from young fluid cultures are non-toxic, while the bodies of the bacilli are highly toxic, but filtrates from old cultures contain a powerful toxin. The death of bacteria occurs also in the body of an infected animal, and the dissolution of the dead bacteria constitutes an important means by which the poisons they contain are set free. There is evidence that during growth bacteria often form poisons which are hurtful to their own vitality, and also that ferments are produced by them which have a solvent effect on the damaged members of the colony. Such a process of *autolysis*, as it has been called, may have an important result in liberating endotoxins. Our knowledge concerning the action of such toxins is chiefly derived from the study of the effects produced by injecting into animals either the bodies of bacteria (killed by chloroform vapour or by heat) or bacterial protoplasm disintegrated mechanically or artificially dissolved. The direct effects produced by such injections in many cases do not present specific characters, though this is in conformity with the fact that in many different infections the symptoms have a certain community in character. The injection of dead bacteria or the products of their autolysis, however, leads to the development of specific antibodies which are associated with the state of immunity (p. 211).

In certain cases there is difficulty in understanding the action of bacteria which neither form soluble toxins in a fluid medium nor possess a highly toxic protoplasm, and which yet produce effects at a distance from the focus of infection, *e.g. B. anthracis*. To explain these occurrences it has long been regarded as a possibility that such bacteria produce toxins only within the animal tissues.

Aggressins.—The evidence adduced for the existence of aggressins as a separate group of bacterial poisons is of the following kind. An animal is killed by a dose of typhoid, dysentery, or cholera organisms introduced into one of the serous cavities. After death the serous exudate which is present is centrifuged to remove the bacteria so far as possible; the remaining bacteria are killed by some antiseptic. Such a fluid, in a dose which by itself has no pathogenic effect, has the property of transforming a non-lethal dose of the bacterium used into one having fatal effect. Further, the effects of the combined actions of the bacteria and aggressins are often of a much more acute character than can be obtained with toxic products developed *in vitro*. The action of *B. anthracis* is

believed to be due to aggressins which are produced only within the body of the host. The effects produced by aggressins have been attributed to a paralysing action on the phagocytic functions of the leucocytes and the reticulo-endothelial system. But there is further evidence that, since they consist of constituents of bacterial bodies, they act by fixing antisubstances and so prevent the latter from opsonizing the bacteria (p. 229). It has been stated also that a special type of immunity against the aggressins can be originated (p. 241). Other workers have pointed out that in the case of certain organisms, products with the properties of the aggressins may be obtained when cultures are extracted *in vitro* with water or serum ; and thus these products are of the nature of endotoxins. The most striking example of a substance with the action of an aggressin is the specific soluble substance of pneumococci. This polysaccharide material is quite non-toxic by itself, but when added to the corresponding anti-pneumococcus serum, it has the property of combining with the latter and so preventing its action on the pneumococci (p. 338). Perhaps the most important aspect of the controversy is the recognition of the existence of toxins having an action on the phagocytes. A poison causing death of leucocytes in connection with the pus-forming action of the pyogenic cocci has been described under the name of *leucocidin*, and Eisenberg records that in *in vitro* mixtures of leucocytes and cultures of the *B. chauvœi*, loss of motility and degeneration of the cells may be observed.

The non-specific effects of endotoxins are responsible for the general changes occurring in the greater number of the common bacterial infections. While aggressins have been spoken of as if they constituted a separate class of toxic substances, this is very doubtful. The term describes merely bacterial products which act by interfering with phagocytosis.

There is another point which must be kept in view, namely, that some of the phenomena which have been regarded as dependent upon the activity of bacterial toxins may possibly be related to the condition of anaphylaxis, an allergic phenomenon (p. 261). Anaphylaxis essentially consists in the development under certain circumstances in an animal of a supersensitiveness to foreign albuminous materials which in themselves are not toxic. Effects of the gravest kind may be produced during this period of supersensitiveness, and it has been thought that some of the phenomena of an infective disease, *e.g.* the intervention of an incubation period before symptoms occur, may be accounted for by the gradual develop-

ment of supersensitiveness to the proteins of the invading bacteria; and it has been demonstrated by methods to be referred to later, that in various infections the tissues of the patient are more susceptible to the products of the invading organisms than are the tissues of a normal individual. The phenomena of any bacterial disease may thus in reality be due to very different and complex causes.

The Nature of Toxins.—There is comparatively little known regarding this subject. The fact that many exotoxins are precipitated along with albumoses suggested the idea that they are formed from the medium in which the bacteria are growing by processes analogous to those of enzyme action. Dernby and Walbum have brought forward evidence in support of the view that toxins are in part formed by the action of enzymes, produced by the organisms, on albumoses contained in the culture medium; thus in the case of the diphtheria bacillus they found that a marked increase of toxicity developed when an extract of the organisms or a small amount of toxin was allowed to act for some time on peptone broth. Further evidence that bacterial toxins are either albumoses or bodies having a still smaller molecule was adduced by C. J. Martin. By filling the pores of a Chamberland bougie with gelatin, he obtained what is practically a strongly supported colloid membrane through which dialysis can be made to take place under great pressure, say, of compressed oxygen. He found that in such an apparatus diphtheria toxin will pass through just as an albumose will.

On the other hand, certain facts indicate that the exotoxins, like the endotoxins, are the product of internal metabolism in bacteria. Thus Brieger and Boer separated from broth cultures of diphtheria and tetanus bacilli, by precipitation with zinc chloride, bodies which showed characteristic toxic properties, but which did not give any of the reactions of proteins, and the nature of which is unknown. This has been confirmed by Hosoya and Miyata using similar methods. It has also been found that the bacteria of tubercle, tetanus, diphtheria and cholera can produce toxins when growing in protein-free fluids. Further investigation is here required, for Uschinsky, applying Brieger and Boer's method to a toxin so produced, states that the toxic body is not precipitated by zinc salts, but remains free in the medium. If the toxins are really non-protein they may, on the one hand, be the final product of a digestive action—extra- or intra-cellular—or they may be the manifestation of a separate vital activity on the part of the bacteria. On the latter theory the toxicity of the toxic albumoses may be due

to the precipitation of the true toxins along with these other bodies.

When we take into account the extraordinary potency of these poisons (in the case of tetanus the fatal dose of the pure poison for a guinea-pig must often be less than 0·000001 gram), we can understand how, altogether apart from their instability, attempts by present chemical methods to isolate them in a pure condition are not likely to be successful, and of their true nature we know nothing.

Of the nature of the endotoxins also nothing is known. They are in general much more resistant to destruction by heat than are the extracellular toxins. On the other hand, in order to produce fatal effects with endotoxins, as a rule much larger doses are required than in the case of exotoxins. It was claimed by Macfadyen that on grinding up typhoid bacilli frozen by liquid air and allowing the material to thaw he obtained the endotoxins in liquid form, and he further stated that by using this fluid he could immunise animals not only against the toxins but also against the living bacteria. It is doubtful, however, whether anti-endotoxins can be developed in animals by injecting endotoxins, although injections of killed bacteria, even in relatively small amounts, may give rise to agglutinating, opsonic, and bactericidal substances in the serum. Thus Friedberger and Moreschi have found that in the human subject the intravenous injection of a fraction of a loopful of a dead typhoid culture gives rise to toxic symptoms, including marked febrile reaction, followed by the appearance of such antibodies.

Vaughan has found that when bacteria are treated with alcohol and ether and then are extracted with alkaline alcohol a " poisonous protein " is split off which he considers is probably a polypeptide [1]; and it is known that substances of this nature have not antigenic properties. If this poisonous protein is really the toxic agent in infections, we would thus have an explanation of the fact that in most instances antitoxins to endotoxins cannot be obtained. He considers that in the procedure followed by him the various secondary and tertiary groups are split off from the toxic molecule, and it is to these groups that the antigenic properties of the bacterial protoplasm, are due, as shown by its giving rise to the other antibodies mentioned. In any case, it seems quite clear that the substances which give rise to the symptoms of general poisoning in in-

[1] Vaughan's protein poison is not obtainable only from bacteria. It can be got from the blood-free tissues of higher animals, and it exerts poisonous action on the homologous species.

fections do not act as antigens, possibly because they are of too simple a constitution or of too small a size. Generally, the more highly susceptible a given animal is to infection with a given bacterium, the more difficult it is to kill that animal with the cellular protein of that bacterium. This is in accord with the view that the resistance of an animal to infection with a given species of bacterium is due to the facility with which its tissues can destroy the organism.

There has been much inquiry regarding the significance of the incubation period which elapses between the introduction of many toxins into the body and the first evidence of consequent damage. This latent period is not invariably present; thus, for example, a fatal dose of staphylococcus toxin when injected intravenously into a rabbit produces its effect within a few minutes. But when tetanus toxin is injected into the substance of the spinal cord so that it comes into immediate contact with the cells on which it acts, there is an interval of some hours before the development of tetanus. It must be noted, however, that action after a latent period is not peculiar to toxins, since the harmful effects of certain physical agencies, such as an exposure to radiations, may develop after a long interval. It has been supposed in the case of toxins that they may act like ferments and initiate some change in the tissues, and that further chemical substances must then be formed before the pathogenic effects are produced. While toxins and ferments may show analogies in their liability to be destroyed by agencies such as heat, there is no close parallelism in the behaviour of the two kinds of bodies. It should be noted especially that toxins differ from ferments in that the former are strictly quantitative in their action

Similar Vegetable and Animal Poisons.—It has been found that the bacterial poisons belong to a group of toxic bodies all presenting very similar properties, other members of which occur widely in the vegetable and animal kingdoms. Among plants the best known examples are the ricin and abrin poisons obtained by making watery emulsions of the seeds of the *Ricinus communis* and the *Abrus precatorius* (jequirity) respectively. From the *Robinia pseudacacia* another poison—robin—belonging to the same group is obtained. The chemical reactions of ricin and abrin correspond to those of the bacterial toxins. They are soluble in water, they are precipitable by alcohol, but being less easily dialysable than the albumoses they have been called toxalbumins. Their toxicity is seriously impaired by boiling, and they also gradually become less toxic on being kept. Both are among the most active poisons known—ricin being the more powerful. When they are injected subcutaneously a period of twenty-four hours usually elapses—whatever be the dose—before symptoms set in. Both tend to produce great inflammation at the seat of inoculation, which in the case of ricin may end in an acute necrosis; in fatal cases hæmorrhagic enteritis and nephritis may be found. Both act as irritants to mucous membranes, abrin especially being capable of setting up most acute conjunctivitis. In the action of a poisonous fungus, *Amanita phalloides*, a similar toxin is at work. After an incubation

period of some hours, symptoms of abdominal pain, diarrhœa with bloody stools, and, later, jaundice occur. *In vitro* the toxin has a hæmolytic action. As in the case of other poisons of this class, an antitoxin can be produced towards the fungus poison.

It is also certain that the poisons of bees, of scorpions, and of poisonous snakes belong to the same group. The poisons derived from the last are usually called venins, and a very representative group of such venins derived from different species has been studied. To speak generally, there is derivable from the natural secretions of the poison glands a series of venins which have all the reactions of the bodies previously considered. Like ricin and abrin, they are not so easily dialysable as bacterial toxins, and therefore have also been classed as toxalbumins. Their properties are also similar; many of them are destroyed by heat, but the degree necessary here also varies much, and some will stand boiling. There is also evidence that in a crude venin there may be several poisons differently sensitive to heat. All the venins are very powerful poisons, but here there is practically no period of incubation—the effects are almost immediate. An outstanding feature of the venins is the complexity of the crude poison secreted by any particular species of snake. C. J. Martin, in summing up the results of many observers, has pointed out that different venoms have been found to contain one or more of the following poisons: a neurotoxin acting on the respiratory centre; a neurotoxin acting on the nerve endings in muscle; a toxin causing hæmolysis; toxins acting on other cells, *e.g.* the endothelium of blood vessels (this from its effects has been named hæmorrhagin), leucocytes, nerve-cells; a toxin causing thrombosis; a toxin having an opposite effect and preventing coagulation; a toxin neutralising the bactericidal qualities of the body fluids and thus favouring putrefaction; a toxin causing agglutination of the red blood corpuscles; a proteolytic ferment; a toxin causing systolic standstill of the excised heart. Any particular venom contains a mixture in varying proportions of such toxins, and the different effects produced by the bites of different snakes largely depend on this variability of composition. The neurotoxic, the thrombotic, and the hæmolytic toxins are very important constituents of any venom. The toxicity of different venoms varies much, and no general statement can be made with regard to the toxicity of different poisons towards man. Lamb calculated that the fatal dose of crude cobra venom for man is probably about 0·015 of a gram, and that if such a snake bites with full gland, many times this dose would probably be injected; but, of courses the amount emitted depends largely on the period which has elapsed since the animal last emptied its glands. When a dose of a venom not sufficient to cause immediate death from general effects is given, very rapid and widespread necrosis may often occur in a few hours round the site of inoculation.

An extremely important fact was discovered by Flexner and Noguchi, namely, that the hæmolytic toxin of cobra venom in certain cases has no action by itself on red corpuscles, but produces rapid solution when some normal serum is added, the latter containing a labile complement-like body, which activates the venom. In this there is a close analogy to what holds in the case of a hæmolytic serum deprived of complement by heat at 55° C. (p. 226). So far no example of the activation of a bacterial toxin is known, but

the results mentioned point to the possibility of this occurring in some cases in the tissues of the body.

There is another group of toxic manifestations which present some analogies to those of the bacterial toxins, but concerning which very little is known. The best example of these is found in the toxic properties of the serum of the eel. If a small quantity of such serum, say 0·25 of a c.c., be injected into a rabbit subcutaneously, death occurs in a few minutes. Although nothing is known of the substances giving rise to such effects, the phenomenon is to be considered as analogous to the action of bacterial toxins

The Theory of Toxic Action.—While we know little of the chemical nature of any toxins, we may, from our knowledge of their properties, group together the exotoxins of tetanus, diphtheria, botulism, etc., ricin, abrin, snake poisons, and scorpion poisons. Besides the points of agreement already noted, all possess the further property that, as will be afterwards described, when introduced into the bodies of suitable animals they stimulate the production of neutralising substances called *antitoxins*; in other words, they act as *antigens*. The nature of the antagonism between toxin and antitoxin will be discussed later. Here, to explain what follows, it may be stated that (1) the toxin forms directly a combination with the antitoxin, and (2) it has been shown that a toxin may lose much of its toxic power and still be capable of uniting with exactly the same proportion of antitoxin. From these and other circumstances Ehrlich advanced the view that the toxin molecule has a complicated structure, and contains two atom groups. One of these, the *haptophore* (ἅπτειν, to bind to), is that by which combination takes place with the antitoxin molecule, and also with presumably corresponding molecules naturally existing in the tissues. The other atom group he called the *toxophore* or *ergophore*, and to this the toxic effects are due. This atom group is brought into relation with the cell elements, *e.g.* the nerve cells in tetanus, by the haptophore group. Ehrlich explained the loss of toxicity which with time occurs in, say, diphtheria toxin, on the theory that the toxophore group undergoes disintegration. And if we suppose that the haptophore group remains unaffected we can then understand how a toxin may have its toxicity diminished and still require the same proportion of antitoxin molecules for its neutralisation. To the bodies whose toxophore atom groups have become degenerated, Ehrlich gave the name *toxoids*. His view with regard to the origin of toxoids from toxins has received ample confirmation within recent years by the work of Ramon and others, and is now generally accepted. The

theory may afford an explanation of what has been suspected, namely, that in some instances toxins derived from different sources may be related to one another. For example, Ehrlich pointed out that ricin produces in a susceptible animal body an antitoxin which corresponds almost completely with that produced by another vegetable poison, robin (*vide supra*). This may be explained on the supposition that the haptophore groups of recin and robin correspond, while their toxophore group differ. The whole subject will be again referred to in the chapter on Immunity.

CHAPTER VI

IMMUNITY

Introductory.—By immunity is meant non-susceptibility to an infective disease or to a pathogenic organism or its toxins. Immunity may be possessed by an individual naturally, and is then usually called *natural* immunity, or it may be *acquired* either by passing through an attack of the disease or by means of artificial inoculation. It is to be noted that man and the lower animals may be exempt from certain diseases under natural conditions, and yet the causal organisms of these diseases may produce pathogenic effects when injected in sufficient quantity. Immunity is, in fact, of very varying degrees, and accordingly the term is used with a relative significance—this is not only true of infection by bacteria, but of toxins also. When the resistance of an animal to a toxin is of high degree, it may nevertheless be overcome by a very large dose. On the other hand, even in cases where the natural resistance is high, this can be still further exalted by artificial means—that is, the immunity may be artificially intensified.

Acquired Immunity in the Human Subject.—The following facts are supplied by a study of the infective diseases which affect the human subject naturally. First, in the case of certain of those, one attack protects against another for many years, sometimes practically for a lifetime, *e.g.* smallpox, typhoid, scarlet fever, etc. Secondly, in the case of other diseases, *e.g.* erysipelas, influenza, gonorrhœa, etc., a patient may suffer from more than one attack. In the case of the diseases of the second group, however, experimental research has shown that in many of them immunity is developed, but is transient; and, though we cannot definitely state it as a universal law, it must be considered highly probable that the passing through an acute attack confers immunity for a longer or shorter period. The immunity is not, however, to be regarded as the result of the disease *per se*, but of the reaction to bacterial products formed in the body during the disease. It has been found in diphtheria, typhoid, cholera, pneumonia, etc.,

that in the course of the disease certain substances appear in the blood, which are antagonistic either to the toxin or to the vital activity of the organism. In such cases a process of immunisation would appear to be going on during the progress of the disease, and when this immunisation has reached a certain height, the disease naturally comes to an end. The immune substances are found in the plasma and the serum; the term "antibodies" has been applied to them. Further, as will be seen, by suitable gradation of the doses of products of the bacteria, or by the use of weakened toxins, a high degree of immunity may be attained without the occurrence of any symptoms whatever.

The facts known regarding vaccination against smallpox exemplify another principle, namely, that immunity may be conferred as the result of passing through a *modified* attack of a disease. We may take it as practically proved that vaccinia is a modified form of smallpox produced by passing the virus through the cow, and that when vaccination is performed, the patient is inoculated with a modified variola (*vide* p. 729). Vaccination produces certain pathogenic effects which are of trifling degree as compared with those of smallpox, and the degree of protection conferred is less complete and lasts a shorter time than that produced by the natural disease.

Artificial Immunity

Varieties.—According to the means by which it is produced, immunity may be said to be of two kinds, to which the terms *active* and *passive* are generally applied.

Active immunity is obtained by introduction into the tissues of (*a*) the organisms either in an attenuated condition or in sub-lethal doses, or (*b*) sub-lethal doses of their products, *i.e.* of their "toxins," the word being used in the widest sense. By repeated injections at suitable intervals the dose of organisms or of the products can be gradually increased; or, what practically amounts to the same, an organism of greater virulence or a toxin of greater strength may be used. The establishment of immunity is commonly attended by the appearance of *antibodies* in the serum, and the constituents of the bacteria or toxins which lead to the development of these are called *antigens*. Such methods constitute the means of *preventive inoculation* or *vaccination*. Immunity of this kind is comparatively slowly produced and lasts a considerable time, the duration varying in different cases.

Passive immunity depends upon the fact that if an animal be immunised to a high degree by the active method, its serum may have distinctly antagonistic or neutralising effects when injected into another animal along with the organisms or with their products, as the case may be; that is, the antibodies developed by active immunisation may be transferred to a fresh animal. Such a serum, generally known as an *antiserum*, may exert its effects if introduced into an animal at the same time as infection occurs or even a short time afterwards; it can, therefore, be employed as a *curative* agent. The serum is also *preventive*, *i.e.* protects an animal from subsequent infection, but the immunity thus conferred lasts a comparatively short time. These facts form the basis of *serum therapeutics*. When such a serum has the power of neutralising a toxin it is called *antitoxic*; when, with little or no antitoxic power, it protects against the living bacterium in a virulent condition, it is called *antimicrobic* or *antibacterial* (*vide infra*).

Immunity toward the various classes of infective agents—bacteria, spirochætes, protozoa, fungi, filter-passing viruses, etc.—appears to depend upon the same principles. Further, the differences which may be presented in the case of different members of any one of these groups are at least as pronounced as those observed between the different groups as a whole.

In the accompanying table a sketch of the chief methods by which immunity may be artificially produced is given. It has been arranged merely for purposes of convenience and to aid subsequent description; the principles underlying all the methods are the same.

Artificial Immunity—Methods

A. Active Immunity—*i.e.* produced in an animal by the introduction, once or repeatedly, of non-lethal doses of an organism or its toxins. It is essential, as a general rule, that the antigen should be *injected into the tissues*, that is, *parenterally*, and not introduced into the alimentary tract.

1. *By the living organisms.*

(*a*) Attenuated in various ways. Examples:

(1) By growing in artificial culture media. (2) By growing at abnormal temperatures, etc. (3) By growing in the presence of weak antiseptics,

or by injecting the latter along with the organism, etc. (4) By passing through the tissues of an animal of different species from the one to be immunised.

(*b*) In a virulent condition, in non-lethal doses.

2. *By the dead organisms.*
3. *By filtered bacterial cultures,* i.e. *toxins ; or by substances derived from such filtrates.*

These methods may also be combined in various ways.

B. Passive Immunity—*i.e.* produced in one animal by injection of the serum of another animal highly immunised by the methods of A.

1. *By antibacterial serum, i.e.* the serum of an animal highly immunised against a particular bacterium in the living and virulent condition.
2. *By antitoxic serum, i.e.* the serum of an animal highly immunised against a particular toxin.

Methods of producing Active Immunity

1. By Living Cultures.—(*a*) *Attenuated.*—In the earlier work on immunity in the case of anthrax, fowl cholera, swine plague, etc., the investigators had to deal with organisms of high virulence, which had accordingly to be reduced before the organisms could be injected in the living state. It is now found most convenient as a rule to start the process of active immunisation with the injection of dead cultures. The principle is the same as that of vaccination, and both attenuated cultures and also the dead cultures used for injection are often spoken of as *vaccines.* The virulence of an organism may be diminished in various ways, of which the following examples may be given :

(1) In the first place, practically every organism, when cultivated for some time outside the body, loses its virulence to a greater or less degree, and in the case of some this is very marked indeed, *e.g.* the pneumococcus. Pasteur found in the case of fowl cholera, that when cultures were kept for a time in ordinary conditions, they gradually lost their virulence, and that when subcultures were made the diminished virulence persisted. Such attenuated cultures could be used for protective inoculation. He considered the loss of virulence to be due

to the action of the oxygen of the air, as he found that in tubes sealed in the absence of oxygen the virulence was not lost. Haffkine attenuated cultures of the cholera vibrio by growing them in a current of air (p. 576).

(2) Many organisms become diminished in virulence when grown at an abnormally high temperature. The method of Pasteur (p. 500), for producing immunity in sheep against anthrax bacilli, depends upon this fact.

(3) Still another method may be mentioned, namely, the attenuation of the virulence by growing the organism in the presence of weak antiseptics. Chamberland and Roux, for example, succeeded in attenuating the anthrax bacillus by growing it in a medium containing carbolic acid in a concentration of 1 : 600. In Calmette and Guérin's method of immunising against tuberculosis a culture of the tubercle bacillus is used which has become highly attenuated as a result of prolonged growth on a medium containing bile.

(4) The virulence of an organism for a particular animal may be lessened by passing the organism through the body of another animal. Duguid and Burdon Sanderson found that the virulence of the anthrax bacillus for bovine animals was lessened by its being passed through guinea-pigs, the disease produced in the ox by inoculation from the guinea-pig being a non-fatal one. A similar principle was applied in the case of swine erysipelas by Pasteur, who found that if the organism producing this disease was inoculated from rabbit to rabbit, its virulence was diminished for pigs. The method of vaccination against smallpox depends upon the same principle (p. 729).

These examples serve to show the principles underlying attenuation of the virulence of an organism. There are, however, still other methods, most of which consist in growing the organism in somewhat unfavourable conditions.

(*b*) *Immunity by Living Virulent Cultures in Non-lethal Doses.*—Immunity may also be produced by employing virulent cultures in small—that is, non-lethal, doses. This may be effected by injecting the organisms into a situation which is unfavourable for the development of the pathogenic action, *e.g.* the intravenous injection of the *Bacillus chauvœi*. In subsequent inoculations the doses may be increased in amount. Such a method, however, is difficult to control, and it has generally been found more convenient to commence the process of immunisation with dead or attenuated cultures, and then to continue with virulent cultures.

Exaltation of the Virulence.—The converse process to attenua-

tion, *i.e.* the exaltation of the virulence, is obtained chiefly by the method of cultivating the organism from animal to animal of the same species—the method of *passage* discovered by Pasteur (first, we believe, in the case of an organism obtained from the saliva in hydrophobia, though having no causal relationship to that disease). This is most conveniently done by intraperitoneal injections, as there is less risk of contamination. The organisms in the peritoneal fluid may be used for the subsequent injection, or a culture may be made between each inoculation. The virulence of a great number of organisms can be increased in this way, the animals most frequently used being rabbits and guinea-pigs. This method can be applied to the organisms of typhoid, cholera, pneumonia, to streptococci and staphylococci, and in fact to those organisms generally which invade tissues. It is known that in cultures the organisms vary in virulence, and this is sometimes associated with differences in colony appearance (p. 39). Within the animal body there may be a survival of virulent strains, although there is also evidence that a true enhancement of virulence in response to environment occurs; in some instances this is indicated by the formation of a capsule. In many instances, however, it is difficult or even impossible to exalt the virulence of an organism by passage.

Depression Immunity.—A somewhat striking phenomenon in active immunisation is that described by Morgenroth, Biberstein and Schnitzer. They found that when mice were the subjects of a chronic general streptococcic infection, the effects of an additional injection of virulent streptococci were diminished; and the important point is that such mitigation was manifest within twenty-four hours after the original inoculation. In some way the first injection had the effect of modifying or depressing the virulence of the organisms used in the second inoculation—hence the term applied by them. The nature of the phenomenon requires further elucidation.

2. Immunity by Dead Cultures of Bacteria.—In some cases a high degree of immunity against infection by a given microbe may be developed by repeated and gradually increasing doses of the dead cultures, the cultures being killed sometimes by heat, sometimes by exposure to some antiseptic, *e.g.* formalin. In this method the so-called endotoxins will be injected along with the other substances in the bacterial protoplasm, but the resulting immunity is chiefly directed against the vital acitvity of the organisms—is antibacterial rather than antitoxic (*vide infra*). It is, however, not universally applicable. The cultures when dead produce, of course, less effect than when living,

and may be conveniently used in the initial stages of active immunisation—to be afterwards followed by injections of the living cultures; this method is extensively used for experimental purposes. This is the method adopted also in man in anti-typhoid and anti-plague inoculations, etc., and for the *treatment* of infections by means of vaccines. When killed cultures are to be used for purposes of immunisation it is important to ensure that the organisms possess the most efficient antigens for developing protective antibodies. As has been seen, variants tend to develop in cultures on artificial media; for example, when the normal type of the colony is "smooth," "rough" forms appear which lack the capacity to produce a solid immunity against the respective infection.

Immunity by Sensitised Dead Cultures.—In this method, which was originally introduced by Besredka, the bacterial suspension is treated with the corresponding antiserum, that is, the serum of an animal immunised against the particular bacterium, and after being left in contact for some time, the serum is separated by the centrifuge and the bacteria are thoroughly washed free of all traces of serum. The bacteria thus treated constitute the *vaccins sensibilisés*. It is claimed that, while immunity produced by them is rapidly developed and is of long duration, the local toxic effects on subcutaneous injection are very much lessened. The method has been applied in vaccination against typhoid, plague, cholera, and dysentery. Apparently in such sensitised vaccines the antigen molecules of the bacteria will be largely combined with antibodies, and thus on theoretical grounds we would expect that only those molecules left free, or those which become free by dissociation, will be able to act as antigens, and thus the antigenic power of the bacteria will be diminished. Certain observations show that this is the case; thus Armstrong found that injections of sensitised pneumococci developed active immunity, but antibody production was less in degree and occurred more slowly than when untreated organisms were injected. It would be desirable to have fuller knowledge of the amounts of antibodies developed by the sensitised and non-sensitised bacteria respectively and of the relation of such amounts to the degree of protection afforded. It is possible that the slow and steady supply of free antigen by dissociation may specially favour the development of the antibody, while the antibody gives a degree of passive immunity.

Combination of Methods.—The above methods may be combined in various ways. By repeated injections of cultures at first in the dead condition, then in the living and attenuated state, and afterwards in the more virulent, and by increasing the doses, a high degree of immunity may be obtained. It is important to note at this point that the most efficient methods of immunisation vary in the case of different infections, but that as a rule a more "solid" immunity is developed by means of

living than of dead cultures. A very valuable procedure consists in injecting living organisms, possessed of some degree of virulence, along with the corresponding antiserum, the latter being either mixed with the bacteria or injected at the same time but in a different situation. The antiserum prevents the occurrence of a severe infection, while the living organisms stimulate effective immunity response. This method is extensively used in the protection of animals. In the human subject it is employed especially in the case of viruses. It is doubtful whether a solid immunity against anthrax and tuberculosis can be developed otherwise than by means of attenuated living cultures. In the case of diseases such as pneumococcal infections (Cecil and Blake), typhoid (Metchnikoff and Besredka), etc., living organisms are more effective than dead organisms as a vaccine. The same is true of many of the filter-passing viruses. Further details will be given in connection with the special diseases.

Vaccines as a Method of Treatment.—Within recent times the principles of active immunity have been directly applied in the treatment of already existing disease. This was originally due to the work of Wright, who employed injections of dead cultures of the causal agents. The justification for such a procedure lies in his contention that in many cases infections are to be looked on as practically localised, *e.g.* the case of an acne pustule, or a boil; and that while the local capacities of resistance may have been lowered, resisting mechanisms in other parts of the body have not been brought into play. The vaccine may thus stimulate these, and the focus of bacterial growth may be flooded with antibodies. It is generally considered that, as a rule, the best results are obtained when an *autogenous* strain of the organism is used, that is, a strain cultivated from the lesion which is to be treated. (With regard to the details of the preparation of the vaccines, see p. 158.) Vaccines have been used extensively in the treatment of acne, boils, sycosis, infections of the genito-urinary tract by the *B. coli*, infections of joints by the gonococcus, and in many cases considerable success has followed the treatment. Favourable results have also been recorded in the case of more general infections, such as ulcerative endocarditis, septicæmia, typhoid fever, etc. In such cases it is stated that the best results are obtained from the use of sensitised vaccines (*vide supra*). In some chronic infections, such as arthritis, benefit has occurred after the *intravenous* injection of typhoid vaccine, which produces a marked febrile reaction, "protein shock." The question arises as to how far this non-specific factor may be responsible in general for the beneficial effects of vaccine treatment.

3. Immunity by Filtered Bacterial Products or Toxins.—The organisms are grown in a fluid medium for a certain time, and the fluid is then filtered through a Chamberland or

other porcelain filter. The filtrate contains the toxins, and it may be used unaltered, or may be concentrated. It is important that the strain of organism used should be one which produces toxin abundantly ; different strains vary greatly in this respect. The process of immunisation by the toxin is started by small non-lethal doses of the strong toxin, or by larger doses of toxin the power of which has been weakened by various methods such as the addition of chemicals, *e.g.* formaldehyde, or by partial neutralisation with antitoxin. Afterwards the doses are gradually increased. This method was carried out with a great degree of success in the case of diphtheria and tetanus. It is capable of general application in the case of organisms where it is possible to get an active toxin from the filtered cultures. It was also applied in the case of snake venoms by Calmette and by Fraser, a high degree of immunity being produced.

The following may be mentioned as some of the most important practical applications of the principles of active immunisation, with a view to the prevention of disease, *i.e.* of protective inoculation: (1) Vaccination of sheep and oxen against anthrax (Pasteur) (p. 500); (2) Jennerian vaccination against smallpox (p. 729); (3) Anticholera vaccination (Haffkine) (p. 575); (4) Anti-plague vaccination (Haffkine) (p. 607); (5) Anti-typhoid vaccination (Wright and Semple) (p. 532); (6) Pasteur's method of inoculation against hydrophobia, which involves essentially the same principles (p. 745); (7) Toxin-antitoxin or toxoid (anatoxin) immunisation against diphtheria (p. 393); (8) Vaccination against Rickettsia infections—Rocky Mountain spotted fever (p. 712); (9) Vaccination against dog distemper (p. 721); (10) Vaccination against yellow fever (p. 783).

Active Immunity by Feeding.—Ehrlich found that mice could be gradually immunised against ricin and abrin by feeding them with increasing quantities of these substances (*vide* p. 196). In the course of some weeks' treatment in this way the resulting immunity was of so high a degree that the animals could tolerate on subcutaneous inoculation 400 times the dose originally fatal. Fraser also found in the case of snake venom that rabbits, by being fed with the poison, could be immunised against several times the lethal dose of venom injected into the tissues. In such cases some of the constituents which act as antigens apparently pass through the intestinal wall unchanged. Vaccines have also been administered by mouth in the case of typhoid and Shiga's dysentery bacillus, but the immunity produced seems to be less than after parenteral administration.

Active immunity of high degree developed by the methods

described may be regarded as *specific,* in the sense explained below.

Local Immunity.—The fact that in the case of erysipelas the infection may be dying out at one place while it is spreading at another, indicates that in the former immunity has been in some way established. According to Besredka, killed cultures of streptococci do not confer immunity when injected subcutaneously but do so when injected into the skin, the larger the number of points of injection the higher being the immunity. Filtrates of cultures are also effective ; they contain " antivirus " which produces immunity only when injected into the skin or applied over the surface. A somewhat similar example of local immunity was observed in the case of infection through the skin in anthrax (p. 501). He finds also that it is possible to produce a local immunity of the respiratory tract against *B. diphtheriæ* by intratracheal injection of the organism in the dead condition ; and of the intestine against *B. paratyphosus* by previous oral administration of ox bile with the killed organisms. In all these cases, immunity is stated not to depend on the presence of antibodies in the blood. They are examples of what he calls the " autonomy " of organs. Investigation of the problem by other workers has indicated, however, that in certain cases local insusceptibility of the skin may be produced by the use of sterile broth instead of antivirus ; again, antibodies are not invariably absent from the serum of animals immunised by Besredka's methods. It has long been known, however, that a certain degree of immunity, or rather of increased general resistance of parts of the body (for example, the peritoneum), can be produced by the injection of various substances—broth, blood serum, solution of nuclein, etc. (Issaeff). These agents probably act by producing a local leucocytosis. Accordingly the insusceptibility to infection which is developed by means of Besredka's procedures would appear to depend partly on local changes involving the reticulo-endothelial system (p. 183), which are non-specific in character, and partly on the production of active immunity in response to the specific antigens introduced.

The Properties of the Sera of Actively Immunised Animals

Antigens and Antibodies.—The fundamental fact in passive immunity, namely, that immunity can be transferred to another animal, shows that the serum in question differs from the

serum of a normal animal in containing antagonistic substances to the bacterium or toxin as the case may be—these being generally spoken of as antibodies. The development of these bodies, first observed in the case of the injection of toxins, is found to occur when a great many different substances are introduced into the tissues of the living body. We can, in fact, divide organic compounds into two classes—(*a*) those which give rise to the production of antibodies, and are thus known as antigens, and (*b*) those which have not this property. Among the former are various toxins, tissue cells, bacteria, red corpuscles, soluble tissue constituents of animal or vegetable origin, etc. They all probably contain a protein constituent, though their true composition is not known, and none of them have been obtained in a pure condition. Among the substances which do not act as antigens may be placed the various poisons of known constitution, glucosides, alkaloids, etc. Though success has been claimed by some workers in obtaining antibodies by the injection of lipoids or complex polysaccharides, these are isolated observations. It may be stated generally that substances of non-protein nature seldom act as antigens, but that certain of such substances when along with protein become antigenic. Proteins in process of hydrolysis rapidly lose their antigenic function, amino-acids and probably also peptones being devoid of the property. We may also state at present that the antibody forms a chemical or physical union with the particular antigen which has led to its development, and the evidence for this will be discussed later. Furthermore, the antibody has apparently a specific combining group which fits, as it were, a group in the corresponding antigen, the two groups having been compared to a lock and key. It is, however, to be noted that this specificity is chemical or physico-chemical rather than biological. An antiserum, for example, developed by the injection of bacterium A may also have some effect on a related bacterium B, and thus appear not to be specific. It is known, however, that the antigens in bacterium A are not all identical, and that some of them may be present, though in smaller proportion, in bacterium B; thus the theory of combining specificity is not invalidated. The number of different antibodies, as judged by their combining properties, would appear to be almost unlimited, a fact which throws new light on the complexity of the structure of living matter.

Specificity.—In connection with the question of specificity the work of Obermayer and Pick and of Landsteiner on modifi-

cation of the antigenic function of protein by chemical means is of great importance. This has been effected by introducing new groups such as iodine, azo- and nitro-radicals, by acetylation or methylation of proteins, etc. Each modified protein acts as a specific antigen and gives rise to a corresponding antibody even in the animal from which the protein was originally derived. For example, Landsteiner and Jablons found that rabbits injected with acetylated horse serum develop antibodies which react specifically with acetylated serum-protein from various other animals (hen, rabbit) in addition to the horse, but not with normal horse's serum or with diazo- or nitro-serum protein. Rabbits also produce an antiserum to acetylated rabbit serum. The serum has thus been deprived of its original species specificity and a new specificity has been acquired, which may be called *structure specificity*. Structure specificity depends on the chemical constitution of the group or compound which has been combined with the serum proteins. This specificity is so delicate that an antiserum to a compound of l-tartaric acid with protein shows little or no reaction with the corresponding antigen derived from d-tartaric acid (Landsteiner and van der Scheer). Such results have an important bearing on Ehrlich's view regarding the mode of formation of antibodies under the influence of antigens, as will be discussed later. The alteration of proteins by physical means such as boiling leads to rather different effects. Boiled protein gives rise to antibodies which react with boiled protein, but only when the latter belongs to the species from which the antigen was obtained ; in this case the species specificity is preserved.

Action of Antibodies.—When antibodies are studied as regards their action *in vivo* or *in vitro* on the substances with which they combine, it is found that (*a*) in certain cases simple combination may occur (*e.g.* antitoxic action), (*b*) in other cases physical effects may be associated with combination (*e.g.* agglutination or precipitation) and (*c*) in a third group of cases the antibody may lead to the union of another body normally present in serum, called complement or alexin. In this third group the combination of complement may or may not result in physical change in the antigen, the evidence of the latter occurrence being elicited by the fixation of complement method (p. 147). Antibodies of the third class are known as immune-bodies or amboceptors (Ehrlich) or sensitising substances—*substances sensibilisatrices* of French writers.

Such are the three classes of antibodies usually recognised, but while the classification is convenient they must not be

regarded as necessarily distinct. It has been recently observed, for instance, that the combination of toxin and antitoxin may be attended by a physical change, namely, flocculation, and it is also known that the antibody which acts as a precipitin is closely related to, if not identical with, the antibody which leads to the fixation of complement.

It is to be noted, however, that the presence of antibodies is not essential to immunity, since after they have disappeared from the blood the animal may still possess immunity. There is thus some change effected in the cells of the body which results in protection, but its nature is not fully known. It has been found, however, that an immunised animal without antibodies in the blood reacts in a different way from the normal animal, inasmuch as antibodies are formed more rapidly and more abundantly on reintroduction of the antigen. But whether this altered power of antibody production is the full explanation of the immunity, is still doubtful. It is certain that in some cases the immunity resides locally in the tissues, *e.g.* in the skin when immunity to erysipelas has been established.

Haptens.—The specific relations of antibodies to antigens can be shown by certain specific reactions *in vitro* to be presently described. As has been stated already, the term antigen should be restricted to a substance which leads to the development of an antibody; there are, however, substances which do not possess the antigenic function but which may still give specific reactions *in vitro*. Such substances are usually known by the name of *haptens*, given to them by Landsteiner. He showed that in the case of antisera developed against proteins combined with various substances of known constitution (*e.g.* organic acids) such substances might display a specific combination with the antibodies developed against the altered proteins, though when introduced into the body the non-protein substances did not act as antigens. Again, in the case of the Forssman hæmolytic antibodies (p. 228) it was found that the lipoidal fraction of the antigen reacted with the corresponding antibody, although on injection into animals it did not lead to the development of antibody. Another example of non-protein haptens is given by the group of bacterial polysaccharides or specific soluble substances to which certain antibody reactions are due. Research on this subject, dating from the work of Dochez and Avery (1917) on the pneumococcus, has shown that such carbohydrate compounds are present in various other bacteria, and that it is to them that the specific characters of antisera to different species or types within a species are due. Thus the

reactions by which different types of pneumococci are distinguished have been shown to be due to combinations of such polysaccharides with protein acting as antigens, while antibodies to the pneumococcus as a species are developed by a protein antigen alone. The compound of polysaccharide and protein resides especially in the capsules of the bacteria, and as capsule formation is often associated with virulence, virulent strains are specially effective in leading to the development of type-specific antibodies. It has thus been clearly shown that certain substances—carbohydrates, lipoids, organic acids, etc. (all non-antigenic in themselves)—when combined with protein confer on the latter the specific antigenic property, and may by themselves give a specific reaction with the corresponding antibody *in vitro*. On the other hand, protein is as a rule essential for the calling out of the antigenic function. Until recently it was believed that the bacterial polysaccharides were quite devoid of antigenic action, but Heidelberger and Goebel have found that if the specific soluble substance of type I pneumococcus is extracted from the organisms by measures which avoid treatment with alkali, some degree of antigenic property is retained. The action of alkali on this substance, however, causes the splitting off of an acetyl group and leads to a product which is non-antigenic, although possessing hapten properties. Raistrick and Topley and others claim also to have obtained from Salmonella organisms antigenic substances of carbohydrate nature which are free from unaltered protein.

After this preliminary statement in explanation, we shall consider the actual properties of antitoxic and of antibacterial sera, and later we shall resume the theoretical consideration.

Antitoxic Serum.—In a previous chapter (p. 189) a distinction has been drawn between exo- and endo-toxins, and with regard to these the general statement may be made that while antitoxins are, as a rule, comparatively easily obtained in the case of the former, the position is quite otherwise in the case of the latter. In certain instances antitoxins to endotoxins have been obtained, but a negative result has been the rule. We have the important fact that in many cases by the injection of dead cultures an active antibacterial serum can be obtained which has no neutralising action on the endotoxins, and we must conclude that many endotoxins are not antitoxinogenic (p. 191). The best examples of antitoxic sera are those of diphtheria, tetanus, and botulism, though similar principles and methods are involved in the case of various other antisera, as well as those to ricin and abrin, and to snake poison. We shall here speak of diph-

theria antitoxin, but the principles and methods described apply to antitoxins generally, although some modifications are required in the case of other antitoxins, *e.g.* for the estimation of the immunity unit, the species of animal used, etc. The steps in the process of preparation are the following: first, the preparation of a powerful toxin; second, the estimation of the power of the toxin; third, the development of antitoxin in the blood of a suitable animal by gradually increasing doses of the toxin; fourth, the estimation from time to time of the antitoxic power of the serum of the animal thus treated.

1. *Preparation of the Toxin.*—The general properties of toxins have been dealt with already; the mode of preparation and the conditions affecting the development of diphtheria and tetanus toxins are described later (pp. 385, 633). The organisms are grown in fluid medium and when the maximum degree of toxicity has been reached the bacillary bodies are removed. The term "toxin" is usually applied for convenience to the bacterium-free product.

2. *Estimation of the Toxin.*—The power of the toxin is estimated by the subcutaneous injection of varying amounts in a number of guinea-pigs, and the minimum dose which will produce death is thus obtained. This, of course, varies in proportion to the weight of the animal, and is expressed accordingly. In the case of diphtheria, in Ehrlich's standard, the minimum lethal dose—known as M.L.D.—is the smallest amount which will certainly cause death in a guinea-pig of 250 grams within four days. This direct method of testing a toxin is a tedious process, because the toxicity diminishes with time. Accordingly it is found more convenient to test toxins by finding how much will be neutralised by a certain amount of a standard antitoxic serum, namely, an "immunity unit" (p. 216).

3. *Development of Antitoxin.*—At first experiments on tetanus and diphtheria antitoxins were performed on small animals, such as guinea-pigs, but afterwards sheep and goats were used, and finally horses. In the case of the small animals it was found advisable to use in the first stages of the process either a weak toxin or a powerful toxin modified by certain methods. Such methods are the addition to the toxin of trichloride of iodine (Behring and Kitasato), the addition of Gram's iodine solution (Roux and Vaillard), and the plan, adopted by Vaillard in the case of tetanus, of using a series of toxins weakened to varying degrees by being exposed to different temperatures, namely, 60°, 55° and 50° C. In recent years formalin (in weak

solution) has been used by Glenny, Ramon, and others, in the early stages of immunisation. By leading to the formation of toxoid (p. 198), it diminishes the toxicity of the toxin without appreciably affecting its antigenic property. In the case of large animals immunisation is sometimes started with small doses of unaltered toxin; and the doses are gradually increased. It has long been recognised that horses vary greatly in their resistance to diphtheria toxin, and also that some of them have a certain amount of antitoxin in their blood under natural conditions. Glenny has found that such animals produce antitoxin rapidly, whilst those with no natural immunity do not respond readily to immunisation. The production of diphtheria antitoxin occurs most satisfactorily when the toxin is injected subcutaneously or intramuscularly; when the intravenous route is used in the later stages antitoxin formation is deficient or may be almost nil (Dean). The antitoxin content of the serum is estimated from time to time, the object of immunisation being, of course, to raise it to as high a level as possible. It is found that each injection produces at first a certain amount of fall in the antitoxin value—the so-called "negative phase," which is only in part due to the neutralising action of the toxin injected—and this, in favourable cases, is followed by a rise to a higher level than before. (Similar phenomena are observed in the development of all other classes of antibodies.) In all cases of immunising, the general health of the animal ought not to suffer. If the process is pushed too rapidly the antitoxic power of the serum may diminish instead of increasing, and a condition of marasmus may set in and may even lead to the death of the animal. The response to the first injection of toxin is slight and occurs slowly; but if a considerable interval is allowed to elapse and then a second dose is given, antitoxin is formed more rapidly and in greater amount than would have occurred had the same dose been given to a normal animal. As Glenny and his co-workers have expressed it, this "secondary stimulus" in an animal which has already developed basal immunity is much more effective than the "primary stimulus"; advantage is taken of this fact in spacing the injections of toxin so as to obtain the best immunity response. The degree of the response depends also on a variety of other factors which have not been completely defined. Non-specific factors such as the addition to the toxin of substances which delay its absorption, *e.g.* tapioca, alum, or lanoline may intensify the response. On the other hand, constituents of the medium in which the toxin

has developed may interfere with the immunity response. After a sufficiently high degree of antitoxic power has been reached, the animal is bled under aseptic precautions, and the serum is allowed to separate in the usual manner. It is then ready for use, but some weak antiseptic, such as 0·5 per cent. tricresol, is usually added as a preservative. Other antitoxic sera are prepared in a corresponding manner. Some further facts about antitetanic serum are given on p. 637.

4. *Estimating the Antitoxic Power of, or "Standardising," the Serum.*—This is done by testing the effect of various quantities of the serum of the immunised animal against a certain amount of toxin. Various standards have been tried, of which Ehrlich's is that now chiefly used. Ehrlich adopted as the *immunity unit* the amount of antitoxic serum which neutralised 100 times the minimum lethal dose of a given specimen of toxin—serum and toxin being mixed together, diluted up to 4 c.c., and, after standing for a short time, injected subcutaneously into a guinea-pig of 250 grams weight; the prevention of the death of the animal for four days was taken as the indication of neutralisation. One c.c. of a serum, of which 0·005 c.c. protected against 100 times the lethal dose, would thus possess 200 immunity units. Sera have been obtained of which 1 c.c. has the value of 2000 units or even more. Within wide limits the principle of constant proportions has been found to hold; thus if the amount of antitoxin is ascertained which neutralises 100 minimum lethal doses of a given toxin, then ten times as much of the same antitoxin will be required in order to neutralise 1000 lethal doses of this toxin. Since the preservation of a standard specimen of toxin was impracticable, Ehrlich employed, as a standard in testing, serum of known antitoxic power which was dried and preserved in a vacuum in a cool place, and in the absence of light. With such a standard test-serum any newly prepared serum can readily be compared.

In order to determine the antitoxic power of a new specimen of antiserum, the following procedure is carried out. In the first place, it is necessary to ascertain the smallest amount of a given toxin which when mixed with the immunity unit of the standard antiserum and injected into a guinea-pig, as above described, will cause death in four to five days: this amount of toxin is called the *limes tötlich* or *Lt dose*. (The precise estimation of this amount can be more readily made than that of the dose of toxin which is exactly neutralised by the antitoxin, so that no symptoms follow on injection.) Then this Lt dose of toxin is mixed with varying amounts of the new specimen of antitoxin, and the mixtures are injected into a series of guinea-pigs; the largest amount of the

serum present in mixtures which lead to death in four to five days is equivalent to one immunity unit.

Economy in animals and rapidity in obtaining results are effected by the *intracutaneous method* of Romer, since one guinea-pig may be injected with a number of mixtures and the results are obtained in two days. The amount of toxin employed in this case is $\frac{1}{500}$ of the L_t dose, that is, the amount which when mixed with $\frac{1}{500}$ immunity unit of antitoxin and injected in an amount of 0·2 c.c. intracutaneously causes a minimal skin reaction ; this is called the L_r dose (Glenny and Allan).

Flocculation.—An interesting physical change resulting from the union of toxin and antitoxin is the flocculation which was discovered by Ramon in the case of diphtheria toxin. He found that when graded doses of toxin are added to a unit of antitoxin, flocculation occurs in some of the tubes. The tube in which this first appears is said to contain the L_f dose of toxin; it is commonly the one in which neutralisation is complete, as tested by injection into a guinea-pig. This flocculation occurs at room temperature, more rapidly at higher temperatures, but in estimating its first appearance it is preferable to work at the temperature of the laboratory. Ramon's results have been confirmed by Glenny and others, and the phenomenon has come to be of service in standardising diphtheria antitoxin, as it supplies a method much more rapid and more easily carried out than any previously in use. But it is necessary to use the animal test in standardising sera for therapeutic purposes, since there may be discrepancies between the latter and the flocculation reaction ; for instance the L_f mixture may sometimes contain excess of toxin when tested by injection into the guinea-pig. The rate at which flocculation occurs varies with different specimens of antitoxin, and it has been suggested by Madsen and Schmidt that sera which flocculate toxin rapidly have a greater " avidity," *i.e.* firmness of combination, than those which act more slowly, and that they will therefore be likely to have a higher curative power. Further investigation of this very important point by Glenny and Barr has indicated that different antitoxin preparations may vary markedly in avidity. This is shown by comparing the action of concentrated and dilute mixtures of the same toxin and antitoxin when a fixed volume (0·2 c.c.) is injected intracutaneously into a guinea-pig. In the case of an antitoxin with weak avidity much more is required to neutralise one L_r dose of toxin when the mixture is dilute (200 c.c.) than when it is concentrated (2 c.c.), whereas when the avidity is high there is no such discrepancy. The avidity as measured in this way, however, did not run

parallel with the rate of flocculation in the Ramon test.

Sera of Animals immunised against Vegetable and Animal Poisons.—It was found by Ehrlich in the case of the vegetable toxins, ricin, and abrin, and also by Calmette and Fraser in the case of the snake poisons, that the serum of animals immunised against these respective substances had a protective effect when injected along with them into other animals. Ehrlich found, for example, that the serum of a mouse which had been highly immunised against ricin by feeding as described above, could protect another mouse against forty times the fatal dose of that substance. He considered that in the case of the two poisons, antagonistic substances—" anti-ricin " and " anti-abrin "—were developed in the blood of the highly immunised animals. A corresponding antagonistic body, to which Fraser gave the name " antivenin " appears in the blood of animals in the process of immunisation against snake poison.

These investigations are specially instructive, as such vegetable and animal poisons, both as regards their local action and the general toxic phenomena produced by them, present, as we have seen, an analogy to various toxins of bacteria.

Nature of Antitoxic Action.—This subject is only part of the general question with regard to the relation of antibodies to their corresponding antigens, but it is with regard to antitoxic action that most of the work has been done. We have to consider here two points, namely, (*a*) the relation of antitoxin to toxin, and (*b*) the source of the antitoxin. With regard to the former subject there is now no doubt that the antagonism between toxin and antitoxin is not a physiological one, but that the two bodies unite *in vitro* to form a compound inert towards the living tissues, there being in the toxin molecule an atom group which has a specific affinity for the antitoxin molecule or part of it.

When toxin and antitoxin are brought together *in vitro,* it can be proved that their behaviour towards each other resembles what is observed in physico-chemical reactions. Thus it has been found that a definite period of time elapses before the neutralisation of the toxin is complete, that neutralisation takes place more rapidly in strong solutions than in weak, and that it is hastened by warmth and delayed by cold. C. J. Martin and Cherry, and also Brodie, showed that diphtheria toxin molecules will pass through a colloid membrane (p. 194), whilst those of the corresponding antitoxin will not. Now if a mixture of equivalent parts of toxin and antitoxin is prepared and is allowed to stand before filtration no toxin is found in the filtrate. Another experiment performed by these workers also proved the occurrence of direct action between toxin and antitoxin ;

they found that a neutral mixture of thermostable snake venom along with thermolabile antivenin became toxic if heated soon after mixing, but when the mixture was allowed to stand for a time and was then heated the toxicity did not return. Again, in cases where the toxin has some definite physical effect, demonstrable *in vitro, e.g.* lysis, agglutination, coagulation, or the prevention of coagulation, its action can be annulled by the antitoxin; in such circumstances manifestly no physiological action of antitoxin through the medium of the cells of the body can come into play. The flocculation resulting from the interaction of toxin and antitoxin is another clear indication of physico-chemical combination.

Although authorities are now agreed as to the direct combination of toxin and antitoxin, there is still much uncertainty as to the exact nature of this union. Regarding this subject there may be said to be three chief views—(*a*) that of Ehrlich, according to which there is a firm chemical union of toxin and antitoxin, and the former is not homogeneous but consists of different molecules; (*b*) that of Arrhenius and Madsen, who considered that the phenomena correspond to the behaviour of two substances in weak chemical union; and (*c*) that of Bordet, who regards the combination to be not of chemical, but of physical nature, corresponding to a process of adsorption.

The Ehrlich Phenomenon and Toxoids.—Controversy on this question may be said to date from the important work of Ehrlich on the neutralisation of diphtheria toxin. Using an immunity unit of antitoxin (the equivalent of 100 doses of toxin) he determined with any example of crude toxin the largest amount of toxin which could be neutralised completely, so that no symptoms resulted from an injection of the mixture. This amount he called the *limes null* dose, expressed as L_o. He then investigated the effects of adding larger amounts of toxin to the immunity unit and observed the quantity which was just sufficient to produce a fatal result, that is, which contained one M.L.D. of free toxin; this amount he called the *limes tötlich*, fatal limit, expressed as L_t. Now if, as he supposed, the union of toxin and antitoxin resembled that of a strong acid and base, $L_t - L_o$ ought to be the equivalent of a minimum lethal dose of the toxin alone. This, however, was never found to be the case, the difference being always considerably more than one M.L.D. This, in brief, is what is known as the "Ehrlich phenomenon," and it was explained by him as the result of the presence of toxoids (*vide* p. 198), *i.e.* toxin molecules in which the toxophore group has become degenerated. He distinguished three possible varieties of such bodies according to the affinity of the haptophore group, namely, *prototoxoid* with more powerful affinity than the toxin molecule, *epitoxoid* with less powerful affinity, and *syntoxoid* with equal affinity. The presence of epitoxoids would manifestly explain the above phenomenon. The L_o

dose would represent toxin + epitoxoid molecules all united to antitoxin molecules, and the presence of another M.L.D. of toxin would not result in there being a free fatal dose, but in the excess of toxin taking the place of epitoxoid. Several lethal doses would need to be added before the mixture was sufficient to produce a fatal result —that is, $L_t - L_0$ equals several M.L.D's.

The main contention of Madsen and Arrhenius is that the toxin-antitoxin combination is not a firm one but a reversible one, and is governed by the laws of physical chemistry. For example, in the case of a mixture of ammonia and boric acid (*i.e.* of a weak base and a weak acid) in solution, there is a constant relation between the amounts of each of the substances in the free condition and the amounts in combination—the combination is reversible, so that if some of the free ammonia were removed a certain amount of the combined ammonia would become dissociated to take its place; further, if to the mixture, in a state of equilibrium, more ammonia or more boric acid were added, part would remain free while part would combine. Accordingly, if toxin and antitoxin behaved in a similar manner, an explanation of the Ehrlich phenomenon would be afforded. Madsen and Arrhenius worked out the question in the case of a great many toxins, and found that the graphic representation of neutralisation is in every case a curve which can be represented by a formula.

According to Bordet's view, the union of toxin-antitoxin does not correspond to that which takes place in ordinary chemical union, but is a physical interaction of bodies in a colloidal state, the action being one of the so-called adsorption phenomena. The smaller toxin molecule becomes entangled as it were in the larger antitoxin one, very much as a dye becomes attached to blotting-paper. He considers also that there is no definite quantitative relationship in the combination of the molecules of the two substances, different amounts of antitoxin being spread over, as it were, and affecting in varying degree, all the molecules of a given amount of toxin. Recent work of Healey and Pinfield, however, has not confirmed Bordet's view. By the use of the flocculation method they found that the amount of diphtheria toxin which combines with one unit of antitoxin, is still capable of fixing one additional unit in presence of excess of antitoxin. But they got no evidence either of the existence of complexes of higher antitoxin content than this or of ones containing less antitoxin than suffices to neutralise the toxin equivalent.

It should be noted in connection with this controversy that there are two questions which may be independent of each other, namely: (1) Does the "toxin" in any particular case represent a single substance or several? (2) What is the nature of the combination of any one constituent substance and its antibody —is it reversible or is it not? It seems impossible to explain the facts with regard to diphtheria toxin on the hypothesis of a single substance, even if this should have its combining and toxic actions equally weakened; "toxoids" in Ehrlich's sense must, in our opinion, be supposed. His results with regard to

the existence of toxoids (epitoxoids at least) have received confirmation from recent workers. Ramon found that if diphtheria toxin was treated with 0·3 to 0·4 per cent. formalin, it lost its toxic action, but still retained its antigenic property and also gave the flocculation reaction as before. A similar change gradually occurs in a toxin when it is kept in the incubator for a prolonged period. He called the detoxicated toxin "anatoxin," but it seems simply to correspond with Ehrlich's toxoids. Glenny and his co-workers confirm the view as to the existence of toxoid; they find that antitoxin has a greater affinity for toxin than for toxoid and may dissociate from combination with either. Then there is an important fact established by Danysz and by v. Dungern, namely, that the amount of toxin neutralisable by a given amount of antitoxin is different according as the toxin is added in several moieties or all at once—in the latter case the amount of toxin neutralisable is greater. Also a given amount of toxin is neutralised by less antitoxin when the latter is added in fractions than when it is added all at once. There seems no explanation of these results according to the view of Madsen and Arrhenius, as the same state of equilibrium ought to be reached in the two cases—that is, the amounts of toxin neutralised should be the same. We have undoubtedly to deal with the combination of two colloids, and the flocculation which may be observed (p. 217) corresponds to a well-known phenomenon in the interaction of colloids. The chief difficulty arises in connection with *specificity*, as this would appear to depend upon the chemical structure and especially the arrangement of radicals, and thus to rest on a chemical rather than a physical basis.

An important factor in the union of toxin and antitoxin is the time necessary for the union to be complete. Morgenroth has shown that in the case of diphtheria toxin this is considerable—about twenty-four hours. Up to this time, mixtures of toxin and antitoxin, when injected intravenously, show decreasing degrees of toxicity according to the time they have been kept. On the other hand, when the subcutaneous method of injection is used the time interval has no effect, and this he considers to be due to a catalytic action of the tissues which accelerates the union of the two substances. He found also that the combination toxin-antitoxin could be broken up by dilute hydrochloric acid and the two constituents recovered; the union is thus reversible. Sachs obtained similar results by means of alkali. A striking phenomenon, which points to the reversibility of the combination, was noted by Behring in the case of diphtheria toxin (later confirmed by Glenny and Barr), and studied by Madsen and by Otto and Sachs in the case of botulism toxin, namely, that when a certain amount of a mixture of toxin and antitoxin was found to be neutral on injection, a fraction of

this amount might produce toxic phenomena or even death. This was apparently due to dissociation of the toxin in the greater dilution, and in favour of this being the case Otto and Sachs found that when the mixture was allowed to stand for twenty-four hours, so that combination was complete, the phenomenon no longer occurred. Other facts might be brought forward which show that the firmness of union of toxin and antitoxin increases with time, or in other words, that dissociation becomes more difficult. There is little doubt that there are varying degrees of firmness of union of an antigen and its antibody, and varying periods necessary for the combination to become complete. It was shown by Morgenroth, and by Muir independently, that the union of a hæmolytic immune-body with the corresponding red corpuscle was of reversible nature ; the latter observer found, however, that in this case the union was not increased in firmness after twenty-four hours.

A statement on the general question is at present impossible ; we can only say that *direct combination of the two bodies does occur ; that sometimes, probably often, the "toxin" contains different toxic bodies with varying affinity ; and that in certain instances the combination has been proved to be reversible, but to what extent this is generally true remains still to be determined. In all cases the outstanding feature is the specific nature of the combination, and of this no satisfactory explanation can as yet be given.*

Antibacterial Serum.—The stages in the preparation of antibacterial sera correspond to those in the case of antitoxic sera, but living, or, in the early stages, dead cultures are used instead of extracellular toxin, and in order to obtain a serum of high antibacterial power it may ultimately be necessary to use a very virulent culture in large doses. For this purpose a fairly virulent culture is obtained fresh from a case of the particular disease, and its virulence may be further increased by the method of *passage.* This method of obtaining a high degree of immunity against the microbe is specially applicable in the case of those organisms which invade the tissues and multiply to a great extent within the body, and of which the toxic effects, though always existent, are proportionately small in relation to the number of organisms present.

The important result obtained by such experiments is, that if an animal be highly immunised by the method mentioned, the development of the immunity is accompanied by the appearance in the blood of *protective* substances, which can be transferred to another animal. The law enunciated by Behring regarding immunity against toxins thus holds good in the case of the living organisms, as was first shown by Pfeiffer. The latter found, for example, that in the case of the cholera organisms, so

high a degree of immunity could be produced in the guinea-pig, that 0·002 c.c. of its serum would protect another guinea-pig against ten times the lethal dose of the organisms, when injected along with them. Here again is presented the remarkable potency of the antagonising substances in the serum, which in this case lead to the destruction of the corresponding microbe. The presence of antibodies in the blood in such actively immunised animals is, of course, a matter of fundamental importance. But it must not be inferred that all the phenomena can thus be explained. For instance, an antiserum to the anthrax bacillus can be developed and by means of it passive immunity can be transferred to another animal; but the preventive property of the serum cannot be explained by the antibodies which can be demonstrated *in vitro*. Similarly, the highly protective effect of an anti-pneumococcus serum cannot at present be satisfactorily correlated with its content in antibodies.

Anti-pneumococcal, anti-meningococcal, anti-streptococcal. anti-dysentery, anti-plague, anti-typhoid, anti-cholera sera, etc., have been prepared. In the case of certain organisms there are several distinct antigenic types, *e.g.* the pneumococcus, meningococcus, streptococcus, dysentery bacillus, etc., and an antiserum to one type has little action, if any, on the other types of the same species.

Properties of Antibacterial Serum.—We have here to consider the three main actions mentioned above, namely, (*a*) bactericidal and lysogenic or bacteriolytic action, (*b*) opsonic action, and (*c*) agglutination and the closely allied precipitation. Of these the two first are those chiefly concerned with the protective property of an antibacterial serum. These various properties are due to the presence of corresponding antibodies in the serum—immune-bodies, opsonins, agglutinins, etc.—but, as already stated, it is not to be assumed that all of these are separate and distinct substances; they are in fact recognisable only by their effects.

(a) Bactericidal and Bacteriolytic Action.—Pfeiffer found that if certain organisms, *e.g.* the cholera vibrio, were injected into the peritoneal cavity of a guinea-pig highly immunised against these organisms, they lost their motility almost immediately, gradually became granular, swollen, and then disappeared in the fluid—these changes constitute what is now generally known as "Pfeiffer's phenomenon" or bacteriolysis. It was subsequently shown by Metchnikoff and by Bordet that bacteriolysis might occur outside the body on the addition of fresh peritoneal fluid or normal serum to heat-inactivated

immune-serum. Pfeiffer also found that an antiserum heated to 70° C. for an hour produced the reaction when injected with the corresponding organisms into the peritoneum of a fresh animal. The outcome of these and subsequent researches was to show that when an animal is immunised against a bacterium, there appears in its serum an antibody, which is generally known as *immune-body*, *amboceptor* (Ehrlich), or *substance sensibilisatrice* (Bordet); it is comparatively stable, resisting usually a temperature of 65° C. for an hour. It cannot produce the destructive effect alone, but requires the addition of a substance normally present in the plasma and serum, which is spoken of under various names—*complement* (Ehrlich), *alexin* or *cytase* (French writers). The complement is relatively unstable, being rapidly destroyed by a temperature of 55° C., and it is not increased in amount during the process of immunisation. Though ferment-like in its instability, it differs from a ferment in being fixed or used up in definite quantities.

Observations by Ferrata, Brand, and others have shown that complement is not a single substance, but is really made up of several components. Thus dialysis or treatment with carbon dioxide or weak hydrochloric acid leads to precipitation of part of the serum globulins. The component in the precipitate, when the latter is separated and dissolved in saline, unites directly with sensitised corpuscles; and then that in the separated fluid enters into combination. Hence they have been called "middle-piece" and "end-piece" respectively. Neither component by itself produces the complementing effect, but together they may act like the original serum. The separation, however, is not always successful. Other components of complement have also been described; serum which has been rendered inactive, *e.g.* with a suspension of yeast cells, may have its complementing activity restored by adding a small amount of serum heated at 55° C. ("third component"), which possesses neither middle-piece nor end-piece properties. Again, Gordon, Whitehead, and Wormall have shown recently that a "fourth component" of complement is destroyed by treating the serum with a minute amount of ammonia, and that reactivation occurs on adding to it a specimen of serum from which the third component has been removed. The work of Browning and Mackie, who fractioned complement-containing serum with ammonium sulphate as well as by treatment with carbon dioxide, has shown that the constitution of complement is even more complex than the above results would indicate. Complement, as tested by bactericidal action, is found to have a similarly complex constitution.

The phenomenon of bacteriolysis is well marked only in the case of certain organisms when an animal is highly immunised against them; the typhoid and cholera group are outstanding examples. It is also to be noted that it is not infrequent in

the case of a normal serum (*vide* Natural Immunity). In other cases the bactericidal effect of a serum may occur without lysis of the bacteria, though other structural changes may be produced. In still other instances, *e.g.* the antisera to staphylococci, streptococci, plague bacilli, etc., a bactericidal effect may be wanting; nevertheless it may be shown that an immune-body is developed in the process of immunisation. This may be done by observing the increased amount of complement which is fixed through the medium of the antiserum (immune-body), sensitised red corpuscles being used as the test for the presence of free complement. The method is described on p. 151.

The all-important action of the immune-body is thus to bring an increased amount of complement into union with bacteria; whether death of the bacteria will result or not will depend ultimately on their sensitiveness to the action of the particular complement.

It is to be noted that in the case of a bactericidal serum there is an optimum amount of immune-body which gives the greatest bactericidal effect with a given amount of complement. If this amount of immune-body be exceeded, the bactericidal action becomes diminished and may be practically annulled. This result, which is generally known as the "Neisser-Wechsberg phenomenon," has been the subject of much controversy, and cannot yet be said to be satisfactorily explained; it is apparently of the nature of a "zone phenomenon" (cf. p. 240). (Regarding some theoretical considerations as to the therapeutic applications of antibacterial sera, *vide* p. 250.)

In certain instances a normal serum may possess bactericidal action on various bacteria, *e.g.* *V. choleræ*, *B. typhosus*, *B. dysenteriæ*, etc., and this may be shown to be due to natural immune-body acting along with complement. In other words, what is observed in the state of active immunity represents a further development of a condition normally present.

The laws of lysogenesis are, however, not peculiar to the case of solution of bacteria by the fluids of the body, but hold also in the case of other cellular elements (red corpuscles, leucocytes, etc.) when these are introduced into the tissues of an animal as in a process of immunisation. Of such sera the hæmolytic have been most fully studied, and, owing to the delicacy of the reaction and the ease with which it can be observed, have been the means of throwing much light on the process of lysogenesis, and thus on one part of the subject of immunity. A short account of their properties may now be given.

Action of Hæmolytic Sera.—It has long been known that in

some instances the blood serum of one animal has, in a certain degree, the power of dissolving the red corpuscles of another animal of different species; in other instances, however, this property cannot be detected. Bordet showed that if one animal were treated with repeated injections of the corpuscles of another of different species, the serum of the former acquired a marked hæmolytic property towards the corpuscles of the latter, the property being demonstrated when the serum is added to the corpuscles. He found that the hæmolytic property disappeared when the hæmolytic serum was heated at 55° C.; but (as with a bacteriolytic serum) lytic action was regained on the subsequent addition of some fresh serum from a non-treated animal. Ehrlich and Morgenroth analysed the phenomena in question, and showed that the specially developed and heat-resisting substance, "immune-body," entered into combination with the red corpuscles at a comparatively low temperature, namely, at 0° C.; whereas complement does not combine at this temperature. In this way a method is supplied by which the immune-body can be removed from a hæmolytic serum while the complement is left. They came to the conclusion that immune-body combines with the complement, though the combination is less firm and only occurs at a higher temperature—best about 37° C. They therefore considered that the immune-body acts as a sort of connecting-link between the red corpuscle and the complement, hence the term "amboceptor" which Ehrlich afterwards applied. It may be stated, however, that the direct union of complement and immune-body has not been conclusively demonstrated. Muir and Browning, for example, found that when a fresh serum is passed through a Berkefeld filter, complement is largely retained in the pores of the filter, whereas immune-body passes through practically unchanged; and that if a mixture of complement and immune-body be made and filtered at a temperature of 37° C., the amount of immune-body which passes through is not diminished, whereas it would be if it had united with the retained complement. Accordingly by this method there was obtained no evidence of the direct union of immune-body and complement. Bordet holds that the immune-body acts merely as a sensitising agent—hence the term *substance sensibilisatrice*—and allows the ferment-like complement to unite. It is quite evident from his writings, however, that he does not mean, as is often assumed, that the immune-body causes some lesion in the corpuscle which allows the complement to act, but simply that it produces in the molecules (receptors) of the red corpuscles an avidity for comple-

ment. All that we can say definitely at present is that the combination antigen *plus* immune-body takes up complement in firm union, while neither does so alone. Even after the corpuscles are laked with water the receptors are not destroyed. Muir and Ferguson showed that they can still take up immune-body and, through its medium, complement, just as the intact corpuscles do. Ehrlich and Morgenroth showed that in some cases the red corpuscles can take up much more immune-body than is necessary for their lysis, and Muir found in one case studied, that each further dose of immune-body led to the fixation of more complement, so that as many as ten times the hæmolytic dose of complement might thus be used up. It is a matter of considerable importance that the union of immune-body and red corpuscles can be shown to be a reversible action. If, as was found by Morgenroth and Muir independently, corpuscles treated with several doses of immune-body and then repeatedly washed in salt solution, be mixed with some untreated corpuscles and allowed to remain for an hour, sufficient immune-body will pass from the former to the latter, so that all become lysed on the addition of sufficient complement. The combination of complement, on the other hand, is usually of very firm nature. It has been a disputed point whether there are several distinct complements in a normal serum with different relations to different immune-bodies, for which Ehrlich and his co-workers have brought forward a certain amount of evidence, or whether, as Bordet holds, there is a single complement, which may, however, show slight variations in behaviour towards different immune-bodies. There is no doubt, however, that all the complement molecules in a serum are not the same. It has been held by workers of the French school that complement does not exist in the free condition in the blood, but is liberated from the leucocytes when the blood is shed; but there are many facts against such a view. For instance, it was shown by Muir and McNee that the introduction of immune-body into the circulation of the corresponding animal leads to a lysis of red corpuscles by means of complement, that the lysis may go on gradually for a considerable period of time and, further, that immune-body may become dissociated from red corpuscles and combine with others, the latter then undergoing lysis. These facts and many others speak strongly in favour of the view that complement exists in the free condition in the circulating blood.

The hæmolytic action of normal serum can be shown in some cases to be of the same nature as that of an immune-serum, that is,

complement and the homologue of an immune-body can be distinguished. For example, guinea-pig's serum is hæmolytic to ox's corpuscles; if a portion of serum be heated at 55° C., the complement will be destroyed; if another portion be treated with ox's corpuscles at 0° C., the natural immune-body will be removed and only complement will be left. Neither portion is in itself hæmolytic, but this property becomes manifest again when the two portions are mixed. Hæmolytic sera are of great service in the study of the question of specificity. Each is specific in the sense already explained (p. 210), but the serum developed against the corpuscles of an animal may have some action on those of an allied species, that is, some receptors are common to the two species. This fact can be readily shown by the usual absorption tests, for example, in the case of an anti-ox serum tested on sheep's corpuscles. A close analogy holds to what has been established in the case of agglutinins. It is, further, of great interest to note that by the injection of red corpuscles into an animal its serum not only becomes hæmolytic, but also in many cases after heating at 55° C. possesses agglutinating and opsonic properties towards the red corpuscles used. These facts show how close an analogy obtains between antibacterial and hæmolytic sera, and how important a bearing hæmolytic studies have on the questions of immunity in general.

Forssman's Hæmolytic Antibody.—It has been shown by Forssman that a lysin for sheep's red corpuscles can be developed in the rabbit by the injection of emulsions of tissues of certain animals, *e.g.* guinea-pig, horse, etc. The antigens in these tissues are known as *heterogenetic* and the corresponding antibodies as *heterophile*. The nature of such antigens is still unknown, though there is evidence that they are lipoidal combinations. The antibodies along with the lipoids obtained by extracting the organs with alcohol give reactions *in vitro*, *e.g.* fixation of complement and flocculation; that is, the lipoid extracts act as haptens. It has been found impossible, however, to obtain heterophile antibodies by the injection of separated lipoids alone; but a mixture of the latter with foreign serum develops in rabbits an antibody which gives reactions with the lipoids (Landsteiner and Simms). Various bacteria contain heterogenetic antigen, *e.g* Shiga's dysentery bacillus.

Cytotoxic Sera.—In addition to hæmolytic sera, antisera have been obtained by the injection of leucocytes, spermatozoa, ciliated epithelium, liver cells, nervous tissue, etc. The laws governing the production and properties of these are identical, that is, each serum exhibits a specific property towards the body used in its production—*i.e.* dissolves leucocytes, immobilises spermatozoa, etc. The specificity is, however, not so marked as in the case of sera produced against red blood corpuscles; thus a serum produced against tissue cells is often hæmolytic; this is probably due to various cells of the body having the same receptors. Here again, when the antiserum produces no destructive effect on the corresponding cells, the presence of an immune-body may be demonstrated by the increased amount of complement which is taken up through its medium. It may also be mentioned that each antiserum usually exhibits toxic properties towards the animal whose cells have been used in the injections, *e.g.* a hæmolytic serum may produce a fatal result, with signs of extensive blood destruction,

hæmoglobinuria, etc, *i.e.*, it is hæmotoxic for the particular animal; a serum prepared by injection of liver cells has been found to produce on injection necrotic changes in the liver in the species of animal whose liver cells were used. These are mentioned as examples of a very large group of specific activities.

(b) **Opsonic Action.**—The presence of a substance in an immune serum which makes the corresponding organism sensitive to phagocytosis was first demonstrated by Denys and Leclef in 1895, in the case of an anti-streptococcal serum. They also showed that the serum produced this effect by acting on the organism, not on the leucocytes. It is, however, chiefly due to the researches of Wright and his co-workers that attention has been directed to the conditions under which phagocytosis of organisms by leucocytes occurs. Wright and Douglas in their first paper showed that the phagocytosis of staphylococci by leucocytes depended on a body in the normal serum which became fixed to the cocci and made them a prey to the phagocytes. To this they gave the name of " opsonin " (*vide* p. 143). There is no phagocytosis of cocci by leucocytes washed in salt solution; normal serum heated to 55° C. is also without effect in inducing this phenomenon. (" Spontaneous " phagocytosis of non-virulent organisms by washed leucoytes in the absence of serum may occur in certain instances, however.) With regard to the mode of action of opsonin, Wright and Douglas could not demonstrate that it had any effect on the leucocytes. On the other hand, if bacteria were exposed to the fresh serum, and they were freed from the excess of serum and then exposed to leucocytes, also washed free from serum, they were readily taken up by the cells. It has been shown that the opsonic action of the serum against an organism is increased by the process of immunisation, and the opsonic index represents the degree of immunity in one of its aspects, as already explained. In an immune-serum, however, an opsonin may still be present after the serum is heated at 55° C., as was shown by G. Dean and others; and Muir and Martin showed that this thermostable immune-opsonin (bacteriotropin of Neufeld) has all the specific characters of antibodies in general. On the other hand, they found that the thermolabile opsonin of a normal serum has quite different properties. For example, when a normal serum is tested on a particular bacterium, the opsonic effect on that bacterium may be removed by treating the serum with other bacteria; in other words, the thermolabile opsonin of normal serum does not possess the specific character of the opsonin developed in the process of immunisation. They found, further,

that various substances or combinations of substances which act as " complement absorbers " also remove the opsonic property from a normal serum, while they have no effect on an immune-opsonin. The opsonic property of fresh normal serum, however, is unaffected by treatment of the serum with certain reagents which abolish complement action, such as ammonia (which destroys the " fourth component " of complement, p. 224), hydrochloric acid, caustic soda or congo red (Gordon, Whitehead, Wormall and Thompson). Accordingly, there is not a complete identity between the action of complement and normal opsonin.

That thermolabile normal opsonin can act in a non-specific way is shown by the fact that particles of carmine and other substances become opsonised by the action of normal serum. It is, however, to be noted that in certain cases there have been found in normal serum traces of substances which can be activated by thermolabile opsonin after the manner of immune-body and complement (as seen in the hæmolytic action of a normal serum, p. 227) ; to this extent the opsonic effect of a normal serum may have some degree of specificity. From this and other facts some observers have attempted to explain the whole of opsonic action according to the scheme of *immune-body plus complement* as seen in hæmolysis. This, however, is not justifiable, since normal thermolabile opsonin can, as we have seen, act by itself, as can also the specific immune-opsonin after normal opsonin has been destroyed by heating. The subject is one of considerable complexity, but it may be said that the most important cause of increase in the opsonic effect observed in infections is the specific bacteriotropins, though the presence of immune-body may play a part by leading to the union of more normal opsonin.

The development of increased opsonic action of the serum may be regarded as the most important factor in active antibacterial immunity, since, as we have seen, an enhanced direct bactericidal action occurs only in the case of a limited number of organisms. The increased action is due to antibodies which act either with or without complement—opsonic immune-bodies or bacteriotropins (immune opsonins) respectively. The latter are of special importance, as they can act in high dilution and are effective in situations where complement has been used up, as is often the case. Moreover, comparative observations show that there is a close correspondence between phagocytosis *in vitro* and *in vivo*. In the immune animal, accordingly, the antibodies lead to ingestion of the bacteria, and this is often, though not invariably, followed by their destruction ; enhanced powers of dealing with the bacteria thus result. Another important factor in relation to phagocytosis is the state of the organisms

as regards virulence. A relatively non-virulent bacterium may be susceptible to phagocytosis when acted on by a normal serum (or even in the absence of serum altogether), but a more virulent strain may require the action of an immune serum; this is seen in the case of certain streptococci. The factors on which virulence depends are various and are imperfectly known, but in some organisms, e.g. *B. anthracis*, an increased virulence is associated with the development of capsules. When anthrax bacilli are introduced into the body there may be active phagocytosis for some time, but then a capsulated race may appear which is not susceptible to opsonic action either in the living body or *in vitro*. Capsule formation in some way protects the bacterium against opsonic action, but increased virulence may occur without it; for example, some strains of virulent streptococci are without capsules, while in others increased virulence is associated with capsule formation. There is also evidence that in some cases virulent bacteria produce aggressins or leucocidins (p. 192) which have an antagonistic action on the phagocytes.

The main facts with regard to phagocytosis in relation to immunity are now recognised to depend upon the opsonic properties of the serum. Metchnikoff, in putting forward his theory of phagocytosis, pointed out the occurrence of phagocytosis in the naturally immune animal and its non-occurrence or feeble occurrence in the susceptible animal, and showed further that the acquisition of immunity against a particular organism was attended by the appearance of active phagocytosis towards it. He explained the phenomena at first by supposing some essential change in the leucocytes, and later, when the favouring action of serum came to be recognised, by supposing that the serum antibodies acted on the leucocytes or, to use his term, acted as "stimulines." Variations in the activity of the leucocytes have been observed in certain conditions, but these are not of specific character, that is, are not specially directed towards the organism against which immunity is acquired. And it is now recognised that, so far as *specific* variations are concerned, the leucocytes are an indifferent factor. All the important variations in phagocytosis in relation to immunity may be said to depend on the opsonic properties of the serum on the one hand, and on the virulence of the organism on the other.

The union of opsonising substance, whether specific or non-specific, has been shown to result in certain physical changes in the bacteria The subsequent phagocytosis of them by the leucocytes occurs with remarkable rapidity, as shown by the fact that several

bacteria may be taken up by a leucocyte within a minute. It is now recognised that both the bringing of the bacteria into relationship with the leucocytes and the subsequent ingestion of them depend on physical processes. The bacteria are drawn together towards the leucocytes by a process allied to agglutination, and thus are seen to form rings around them (Ledingham). Increased adhesiveness of the bacteria to the leucocytes is produced by opsonic action, and this means diminished surface tension at the point of contact, the bacteria thus becoming merged in the leucocyte protoplasm quite apart from amœboid activity. Recently tannic acid has been found to opsonise organisms, and substances in general which possess tanning properties, act in this way (Gordon and Thompson). These facts have been established with regard to *in vitro* experiments, but the same principles no doubt hold with regard to phagocytosis in the body. At the same time it must be recognised that leucocytes in the tissues move towards the bacteria under chemotactic attraction, and their emigration from the vessels seems capable of explanation only in this way. Such chemotactic attraction has been demonstrated also *in vitro* in certain instances.

Summary of the mechanism of opsonic action

(1) *Pathogenic organisms are usually not phagocytosed by leucocytes in the absence of serum.*

(2) *Opsonins, the substances in serum which promote phagocytosis, act by altering the organisms; they do not have any direct effect on the leucocytes.*

(3) *The opsonins of normal and immune sera may be distinguished.*

(4) *Normal opsonins are thermolabile at* 56° *C. They are non-specific, since treatment of unheated serum with one species of organisms removes the opsonins for other species; they are related to, but not identical with, bacteriolytic and hæmolytic complement. Thus many procedures which remove the complementing action of serum also deprive it of normal opsonin; on the other hand, serum which has been deprived of the fourth component of complement, still has opsonic action.*

(5) *Immune opsonin (bacteriotropin), which is developed in the serum as the result of immunising an animal with the organism in question, has the properties of a specific thermostable antibody.*

(6) *The opsonic effect of complement* plus *immune opsonin may be greater than the sum of the opsonic actions of each component separately.*

(7) *In certain cases the opsonic property of a normal serum is due to thermostable constituents.*

(c) **Agglutination.**—Charrin and Roger in 1889 observed that when *Bacillus pyocyaneus* was grown in the serum of an animal immunised against this organism, the growth formed

a deposit at the foot of the vessel; whereas a growth in normal serum produced a uniform turbidity. Gruber and Durham, in investigating Pfeiffer's reaction, found that when a small quantity of an antiserum is added to a suspension of the corresponding bacterium, the organisms become agglutinated into clumps, this phenomenon depending upon the presence of bodies in the serum called *agglutinins*.

It had already been found that the serum of convalescents from typhoid fever could protect animals to a certain extent against typhoid infection, and, in view of the facts experimentally established, it appeared a natural proceeding to inquire whether such serum possessed an agglutinative action and at what stage of the disease it appeared. The result, obtained independently by Grünbaum and Widal, but first published by the latter, was to show that the serum possessed this specific action shortly after infection had taken place; in other words, the development of this variety of antibody can be demonstrated at an early stage of the disease. Agglutination may be said to be observed generally in bacterial infections, though the degree of the phenomenon and the facility with which it can be noted vary greatly in different cases. Details will be found in the chapters dealing with individual diseases, etc. Furthermore, the phenomenon is not peculiar to bacteria; it is seen, for example, when an animal is injected with the red corpuscles of another species, *hæmagglutinins* appearing in the serum, which have a corresponding specificity, as was first shown by Bordet.

Agglutination has usually been regarded as a phenomenon which in itself does not play any part in conducing to the state of immunity. In the case of rabbits whose blood was richly infected with pneumococci, Bull, however, found that the intravenous injection of anti-pneumococcal serum of homologous type from a horse brought about immediate clumping of the cocci and their phagocytosis by polymorphonuclear leucocytes in the capillaries of the liver, spleen, and lungs. He concluded that the agglutination is an essential process along with the phagocytosis in bringing about protection. The administration of excessive doses of antiserum, however, caused the formation of clumps of the cocci so large that they escaped phagocytosis and thereupon multiplied, causing a fatal infection. The agglutinating action of antibody appears also to account for the fact observed by Rich that pneumococci injected into the skin of an immunised rabbit remain localised, whereas in a susceptible animal they rapidly spread through the tissues.

Varieties of Agglutinins.—It has now been shown by the work

of Weil and Felix and others that in the case of many motile bacteria agglutinins are developed against both the flagella and the bodies of the bacteria—*flagellar* or H, and *somatic* or O agglutinins respectively, and these two types have a different significance. The flagellar agglutinins lead to a rapid formation of loose flocculent clumps, while the somatic agglutinins act more slowly and the clumps are smaller and denser. The somatic antigens or agglutinogens are demonstrated in the case of motile bacilli by means of non-motile variants or by the use of suspensions which have either been heated at 100° C. or treated with alcohol—these procedures having the effect of lysing the flagella (Craigie). The flagellar agglutinins have a slightly greater resistance to heat than the somatic. In the process of immunisation against motile organisms flagellar agglutinins appear first, and in a well-developed antiserum flagellar agglutination occurs with a much higher serum dilution than somatic agglutination. In a corresponding way, in vaccination with such organisms, *e.g.* with *B. typhosus,* flagellar agglutination becomes marked, whilst somatic agglutinins are absent or only in low titre. In the coli-typhoid group the flagellar agglutinins appear to be specific and are thus of most importance in the diagnosis of infection, provided that the necessary precautions be taken. The somatic agglutinins of related bacteria may on the other hand be the same, *e.g.* the O agglutinin of *B. typhosus* and that of *B. enteritidis*. In other groups, however, the converse may obtain, the O agglutinins being more specific than the H agglutinins. It is maintained by some, *e.g.* Felix, that the somatic agglutinins are the same as the bactericidal and complement-fixing immune-bodies; at any rate they are more closely related to these than are the flagellar agglutinins and accordingly constitute a better index of true immunity. In addition to the somatic and flagellar agglutinins one has also to add that the agglutinins to the rough and smooth variants of certain organisms differ from one another (p. 38). The receptor analysis of bacteria, especially when motile, comes thus to be a complicated matter, and the conditions for making a diagnosis by means of agglutinins must be worked out in the case of each infection.

Vi Antigens and Antibodies.—Another point, of which the practical importance has long been recognised, is that bacteria when freshly grown from the tissues are often less agglutinable than they afterwards become when subcultured for some time. Felix and Pitt (1934) showed that living suspensions of different smooth strains of *B. typhosus* vary greatly in their agglutin

ability by the somatic agglutinins and that the inagglutinable strains are the most virulent on testing in mice. They were able to relate the high virulence and inagglutinability to a special antigen possessed by the strains in question. The separate identity of this *virulence* (*Vi*) *antigen* was shown by agglutinin absorption; by removal of the antibodies to the H and O antigens from an antiserum containing a mixture of all three antibodies, a preparation can be obtained which exhibits anti-Vi action alone. One property of the Vi antigen is to render the somatic agglutinogen resistent to the agglutinating effect of the corresponding antibody and also to its opsonic and bactericidal action. The Vi antibody agglutinates only those organisms which contain Vi agglutinin, also it promotes their phagocytosis; and it confers passive immunity on mice against inoculation with virulent typhoid bacilli. It is, however, not the sole protective antibody in antityphoid serum, since it is inferior to an anti-O-serum in protecting against infection with organisms of low virulence or the toxic effects of killed cultures. The Vi antigen, which is specific for the typhoid bacillus, is formed only where the organisms grow under particular conditions, *e.g.* not below 25° C. or above 40° C.; further, it can be demonstrated in its most characteristic form only in living organisms and it is readily destroyed by various agencies, *e.g.* phenol or heat, although it may partially resist 100° C. After prolonged subculturing in artificial media the Vi antigen may still be formed and it has been found in non-virulent rough variants. A similar antigen has been detected in Salmonella bacilli.

In addition to its value in the *diagnosis of infections*, the agglutination reaction is of great service in *the differentiation of bacteria*; in this case the agglutinability of an unknown bacterium is tested by a known antiserum. If the unknown organism is agglutinated by the antiserum in the same dilution as the homologous organism, this is evidence of their identity. One must recognise, however, that occasionally different pathogenic organisms react in the same way to an agglutinating serum and cannot be differentiated by this method. For example, in the three following instances, *Gonococcus* and *Meningococcus*; *B. melitensis* and *B. abortus*; and some strains of *B. mallei* and *B. whitmori*, it is recognised that the two organisms usually cannot be distinguished by direct agglutination. In the Salmonella group also, different members may be agglutinated to the same titre as the homologous organism by an antiserum. In such cases of co-agglutination differentiation of the cultures

can be effected by the method of agglutinin absorption—Castellani's reaction (see p. 141). The principle is that the homologous organism will remove from an antiserum the agglutinins both for itself and for the heterologous organism, whereas the latter will remove from it only the group-agglutinins. Accordingly, if an antiserum to culture X agglutinates culture Y, then tests made with the following results indicate that Y is a heterologous organism :

Anti-X Serum absorbed with	Agglutinating Action of the Treated Serum on	
	Culture X	Culture Y
Culture X . .	nil	nil
Culture Y . .	practically unchanged	nil.

A further refinement consists in using anti-H or anti-O sera, etc. In this way the identity or otherwise of the flagellar or somatic agglutinogens may be established. It has been supposed that the different species of organisms each contain a "mosaic" of antigens and hence that the group reaction is due to certain antigens being common to both. This explanation is questioned, however, by Burnet. Identification of a bacterium can thus be made under certain conditions, and related organisms can be distinguished by the absorption method.

Another point is that where there has been formation of agglutinins, either as the result of inoculation or of an infection, and the agglutinins have disappeared from the blood, a re-stimulation of their formation may be produced by various febrile diseases. This non-specific stimulation is manifestly of importance in diagnosis. Felix has found in the case of the typhoid-paratyphoid group that it is the H or flagellar agglutinins that are affected in this way. Thus in a patient at some time previously inoculated with a vaccine such as T.A.B., a reappearance of the corresponding agglutinins may result from another febrile attack. On the other hand, he has found that the O or somatic agglutinins are not liable to this non-specific stimulation. Accordingly, when they are present they have a diagnostic significance (p. 530).

It was shown by Kraus and by Nicolle that if an old bacterial culture be filtered through porcelain the addition of some of the corresponding antiserum produces a sort of granular precipitate (*vide infra*) in it, and that if minute inorganic particles or heterologous bacteria are added to the mixture they become aggregated into clumps as in the agglutination of the homologous bacteria In this case the clumping depends on and is secondary to the process of precipitate formation, and is thus of somewhat different nature from agglutination in saline solutions.

Besides those stated above, other phenomena have been observed in the interaction of antisera and the corresponding bacteria. For example, it has been shown that when certain bacteria—*e.g.* the typhoid bacillus, *B. coli*, and *B. proteus*—are grown in broth containing a small proportion of the homologous antiserum, their morphological characters may be altered, growth taking place in the form of threads or chains which are not observed in ordinary conditions. In other instances a serum may inhibit some of the vital functions of the corresponding bacterium.

The *adhesion phenomenon of Rieckenberg* observed with trypanosomes and spirochætes appears to be allied to agglutination in nature. When the uncoagulated blood of a mouse which has been cured of trypanosome infection is added to a suspension of the homologous parasites the latter quickly become coated with blood platelets. Similarly if living *Sp. recurrentis* are mixed with the corresponding antibody and bacteria are added the latter adhere to the spirochætes.

Precipitins.—Shortly after the discovery of agglutinins, Kraus showed in the case of the organisms of typhoid, cholera, and plague, that the antiserum not only caused agglutination, but when added to a filtrate of a culture of the corresponding bacterium, produced a cloudiness and afterwards a precipitate. To the substance in the immune-serum which brought about this effect he gave the name of *precipitin.* Subsequent study has shown that this phenomenon is closely related to agglutination. To test the reaction it is accordingly necessary to have as far as possible constituents of the bacteria in solution, and for this purpose there have been introduced various methods, of which the two following may be given :

(*a*) It is well known that in an old broth culture the bacteria undergo disintegration and their constituents go into solution. Accordingly, if such a culture which has been kept in the incubator for several weeks be filtered through a porcelain filter, the filtrate will contain the reacting substance or precipitinogen.

(*b*) The growth from a recent agar culture is scraped off and suspended in normal salt solution, the mixture is made feebly alkaline with soda solution and boiled for a few minutes. The mixture is then neutralised, when a precipitate forms, and is filtered through filter paper ; the filtrate contains the precipitinogen.

A method which is very delicate is the ring test ; it consists in floating varying dilutions of the bacterial extract on to the surface of a fixed amount of antiserum in a small tube. The occurrence of precipitation is shown by the appearance of a ring of opacity at the junction of the fluids. In eliciting the agglutination and precipitation reactions there is in general a noteworthy difference in procedure ; in the former a minute amount of antiserum acts on a large quantity of the organisms, whereas precipitation is obtained when an excess of antiserum

is mixed with a small amount of antigen. This precipitin reaction has now been observed in a great many bacterial diseases when the patient's serum is added to the corresponding bacterial filtrate, and has even been applied by some observers as a means of diagnosis. It is, however, more restricted in its application than the agglutination methods. The reaction is applied in the diagnosis of anthrax infections by demonstrating the presence of antigens of the anthrax bacillus in extracts of an animal's organs (p. 503). The reaction may also be given by haptens (p. 212) along with the homologous antisera, for example, the specific soluble substance of pneumococci (p. 342) or the carbohydrate-containing hapten extracted from tubercle bacilli by Laidlaw and Dudley (p. 438). In Lancefield's method of investigating the serological relations of streptococci, the precipitinogen is extracted from the organs with acid and heat.

Serum Precipitins.—This subject does not strictly belong to bacteriology, but the general phenomena are so closely allied to those just described that some reference may be made to it. When the serum of an animal is injected in repeated doses into another animal of different species, after the type of an immunisation, there appears in the serum of the animal treated a precipitating antibody (precipitin), which causes a cloudiness or precipitate when added to the serum (precipitinogen) used. (In the case of rabbits, doses of 3 to 4 c.c. of the serum may be injected intraperitoneally at intervals of four to five days, a precipitin usually appearing at the end of about three weeks. Dean obtained the best precipitins by giving six to eight injections each of 2 c.c. of serum at intervals of five days—the first few being intravenous and the rest intraperitoneal—and then after a rest period of three to six months a second series of injections.) The reaction, which is a very delicate one, is conveniently observed by adding a given amount of the antiserum, say 0·05 c.c., to varying amounts of the homologous serum 0·1, 0·01, etc., c.c., in a series of small test-tubes, the volume being then made up with salt solution to 1 c.c. In this way a definite reaction may be observed with 0·0001 c.c. of the homologous serum or even less. An even more delicate reaction is obtained when the solution of antigen is placed with a pipette on the surface of the solution of precipitin, a layer of cloudiness then forming at their junction—the so-called "ring" test. Here again zone phenomena, as in the case of agglutination, are met with. If the antiserum be heated to a temperature of 75° C. for some time it acquires inhibitory properties, so that when added to a mixture of serum and antiserum which would otherwise give a precipitate, this no longer occurs. Some observers consider that this is due to the presence of "precipitoid" in the heated antiserum; but the observations of Welsh and Chapman show that this view is not in accordance with the facts, and indicate that the inhibition is related to a specific solvent action which the heated antiserum has on the precipitate. They have also shown that the main mass of the precipitate is furnished by the antiserum (precipitin), and not as was usually

supposed by the protein of the homologous serum thrown down by the precipitin; this result is of high importance in connection with the action of antibodies in general. The precipitin reaction is specific in the sense explained above. It is always most marked towards the serum of the species used in the immunisation; but while this is so, there may also be a slight reaction towards animals of allied species. An anti-human serum, for example, gives the maximum reaction with human serum, but also a slight reaction with the serum of monkeys, especially of anthropoid apes; it however, gives no reaction with the serum of other animals. The precipitin test has thus come to be employed as a means of differentiating human from other bloods. For critical quantitative determinations of either antigen or antibody Dean employs a method for determining optimal proportions, *i.e.* those in which the most rapid formation of particles occurs. First, a rough test is performed in which the following antiserum dilutions—1 : 5, 1 . 10, 1 : 20, 1 : 40—are each mixed with a series of eleven dilutions of antigen ranging from 1 10 to 1 : 10,000. The concentration of antiserum which yields precipitation in a convenient time is noted and also the dilution of antigen with which this occurs. Then by mixing 1 c.c. amounts of this concentration of antiserum with a series of 1 c.c. volumes of varying dilutions of the antigen, each differing by 0·00025 c.c of serum, the optimum is ascertained

Another interesting phenomenon, the fixation of complement reaction, is produced by the combination of the two substances in the serum (antigen) and antiserum (antibody) respectively. If mixtures be made according to the first method mentioned above, and then a small quantity of complement (fresh guinea-pig serum) be added—say slightly over one M.H.D. in the absence of the antigen—it will be found that the complement becomes absorbed, as may be shown by subsequently adding a test amount of sensitised red blood corpuscles. This fixation phenomenon is even a more delicate reaction than the precipitin test, it being often possible to demonstrate by its use from a tenth to a hundredth of the smallest amount of serum which will give a perceptible precipitate; it also is specific within the same limits.

Mechanism of Agglutination and Precipitation.—The physical changes on which agglutination depends cannot as yet be said to be fully understood. Two stages can be distinguished in the phenomenon: (*a*) the combination of agglutinin and agglutinable substance (agglutinogen), and (*b*) the actual clumping of the bacteria. For the occurrence of the latter phenomenon an essential factor is a suitable salt content. Bordet showed that when the bacterial emulsion and the agglutinin are made salt-free by dialysis, agglutination does not take place on their being mixed, although the agglutinin is fixed by the bacteria. On the addition of an electrolyte such as sodium chloride agglutination occurs. Further, if subsequently the clumps of agglutinated bacteria are again freed from salt by washing in distilled water they become resolved; but on the

addition of some sodium chloride they are formed again. It is also to be noted that many strains of bacteria, *e.g.* the rough forms of the coli-typhoid group, agglutinate in salt solutions without the presence of an antibody—spontaneous agglutination. Various bacteria may also be agglutinated by acids, there being an optimum hydrogen-ion concentration which varies in the case of different bacteria.

A further important point is that the occurrence of agglutination after the combination of agglutinin and agglutinogen depends on the physical condition of each, as well as on the presence of electrolytes. For example, in some cases when the bacteria are heated at a temperature of 65° C. for some time, they may lose the faculty of being agglutinated while they may still retain the property of combining with or binding agglutinin. Dreyer and Jex-Blake have observed the remarkable fact that in certain instances on being heated to a still higher temperature they may once more become agglutinable.

It is agreed that agglutination and precipitation are closely allied phenomena and present certain similarities to well-known reactions exhibited by colloids. In both there is a reduction in the dispersion of colloid particles, these being in agglutination, the bacteria in suspension, and in precipitation the colloidal particles of the bacterial extract or the protein in solution. It is recognised that the chief force which keeps particles or molecules apart in a fluid is the like electric charge which they bear, while surface tension is the force which tends to draw them together. Experiments on the electrical state of *B. typhosus* when suspended in weak neutral saline solution have shown that the organisms carry a negative charge ; a reduction of this charge will tend towards their adhesion. A kation such as sodium reduces the surface charge only when in relatively high concentrations and does not agglutinate the bacilli unless they are combined with agglutinin. Another striking resemblance to colloidal interaction is seen in the *zone phenomena*. For example, when the amount of precipitin is kept constant in a series of tubes and gradually increasing amounts of precipitinogen (antigen) are added, the resulting precipitate increases up to a point, but beyond that point increased amounts of antigen cause diminution of the precipitate, and ultimately its disappearance. A zone phenomenon is also observed with agglutinating antisera—absence of agglutination in slightly diluted serum, and agglutination in higher dilutions till an end-titre is reached. Antisera which have been heated to 60° to 70° C., and in which the agglutinins have become modified, tend especially to

show the zone phenomenon. A similar zone phenomenon is seen when, for instance, increasing amounts of a colloidal solution of iron hydroxide are added to a mastic emulsion. This phenomenon has been explained as being due to alteration of the electric charge of the particles of the one colloid by the added particles of the other, till the charge is brought to zero; there then occurs the maximum precipitate. Further addition leads to reversal of the charge, and thus once more to dispersion of the particles.

Even if it is the case, as seems certain, that the physical phenomena of agglutination and precipitation correspond with those in the interactions of other colloids, it must be borne in mind that the essential factor is the *specific* combination of antigen and antibody—of agglutinogen and agglutinin, of precipitinogen and precipitin; and to this there is no analogy in colloidal reactions so far as we know. Interesting results have recently been recorded by Topley and others, which suggest that in addition to the factors mentioned there is some specific affinity between antibody-laden organisms belonging to the same species. This appears from the observation that when mixtures of two species of organisms, *e.g.* pneumococci and typhoid bacilli, are agglutinated by a mixture of the corresponding antisera, the clumps which form are composed practically wholly of the one kind of organism or the other. Non-protein carbohydrate constituents may be the agglutinogens responsible for the serological characters of bacteria. Thus in the case of the cholera vibrio, according to Bruce White there may be present in the S-form four such components, the suppression of one or more of these being associated with the formation of variant types of the organism.

Antiaggressins.—In the case of certain organisms such as *B. anthracis*, the serum of actively immunised animals does not manifest *in vitro* any of the properties of antibodies described above; nevertheless it confers passive immunity, but it is not readily deprived of its protective action by treatment with the homologous organisms in the test-tube. Bail has attributed the action of such antisera to antiaggressins. Experimental evidence in support of this view has been furnished by Pettersson, who has shown that if anthrax aggressin and virulent anthrax bacilli be mixed with normal rabbit serum in one case and in the other with anti-anthrax immune serum, and the mixtures are then injected subcutaneously into a normal rabbit, different results follow. At the site of injection of the mixture containing immune serum leucocytes collect in abundance, but where the mixture containing normal serum was injected

leucocytes are absent. Accordingly, the antiserum appears to inhibit the action of aggressin in preventing accumulation of the leucocytes.

Unity or Plurality of Antibodies.—In the above account we have spoken of different antibodies, but, as already stated, the names used merely indicate the possession of different properties by antisera as revealed by interaction with antigens. There has been, and still is, dispute as to whether these properties correspond with different substances or whether all the properties are borne by one—the "pluralistic" and "unitarian" views respectively. In the first place, we may say that there is no justification for inferring the existence of a particular substance corresponding to each reaction. In all cases the union of the antibody causes some change in the bacterial antigen, and this may result in various effects according to the method of test. Thus we may have agglutination, precipitation, increased adhesiveness and opsonic action, fixation of complement, which in its turn may lead to the death of the bacterium or to its being opsonised, or, lastly, to no observable effect. All these effects may possibly be produced by the same antibody, and the close relation between precipitation and complement fixation has already been shown. Frequently the obvious manifestations of the interaction between antigen and antibody depend on the presence of some third agent which, as it were, acts as an indicator. Thus, Hartley found that antitoxic serum from which the lipoids had been extracted, did not flocculate the corresponding toxin, although it still neutralised the toxic effects as tested by injection into a susceptible animal. On the other hand, an antityphoid serum which had been similarly extracted still agglutinated typhoid bacilli in as high dilution as the untreated serum, while in the latter reaction the essential part played by electrolytes in flocculating the agglutinin-laden bacteria has already been mentioned. It does not follow from what has been stated above, however, that all the antibody molecules, if one may speak of such, are similar in the sense that all the properties revealed by reactions are held in the same proportion. The study of the various antibody reactions in the course of development of antisera shows considerable variations in the properties at different stages, and it does not seem possible to reconcile this with the idea of a single antibody. And it has been found in the case of hæmolytic serum that an immune-body itself shows qualitative changes at different stages. It would therefore appear that antibodies may differ in other respects than in their combining groups; that is,

they are not uniform in their reacting properties. On the other hand, various reacting properties may be held by the same antibody. A further point which must be considered in regard to the action of an antiserum, *e.g.* to a bacterium, is that the latter may contain a multiplicity of antigens, each of which may lead to the development of a corresponding antibody. The flagellar and somatic antigens of the typhoid bacillus illustrate this. According to Felix it is the somatic antibody which is most active in leading to fixation of complement. Again, different animal species may respond to the injection of the same antigen by the production of antibodies which are not identical in their properties.

The Source and Nature of Antibodies.—The earlier work on these questions was concerned mainly with antitoxins, and various theories were put forward. One of the first views to be advanced was that antitoxin molecules represented toxin molecules which had been in some way modified by the cells of the body; but it was soon established that this view could not be maintained. It was found that the amount of antitoxin produced by an animal may be many times greater than the equivalent of toxin injected; and, further, that when an animal is bled the total amount of antitoxin in the blood may some time afterwards be greater than it was immediately after the bleeding, even although no additional toxin is introduced. The latter circumstance shows that antitoxin is *formed* by the cells of the body. Recent work has fully confirmed the view that the antigen does not enter into the composition of the antibody. For instance, in the case of an antigen composed of the arsenical drug atoxyl diazotised and combined with foreign protein, the resulting antiserum derived from a rabbit, when tested in a considerable quantity contained no demonstrable amount of arsenic (Berger and Erlenmeyer). This being so, it remains to be determined whether the antibody is a normal constituent of the cells which is formed in increased quantity or whether it is a new product. The presence of representatives of a great multiplicity of antibodies in normal sera is a circumstance of great significance, as these are undoubtedly the products of cellular activity, and in all probability molecules of corresponding nature occur as constituents of cells; an increased formation and setting free of these may therefore explain the production of antibodies in active immunity. It should be noted that many of these antibodies act on antigens with which the animal's tissues cannot possibly have come into previous contact. But have all the antibodies normal

representatives? If not, then the molecules of antigen must by a sort of impress alter the configuration of molecules in the cells so that they function as antibodies. Regarding such an occurrence, however, we know nothing. As to the manner in which the antigens influence the cells, we have evidence that in the living body bacterial toxins enter into combination with, or, as it is often expressed, are fixed by the tissues—presumably by means of certain combining affinities. This has been shown by the experiments of Dönitz and of Heymans with tetanus toxin. In such cases there is no evidence as to where the toxin is fixed beyond that supplied by the occurrence of symptoms. We may note, however, that it is not a serious objection that in certain animals other tissues than that of the central nervous system can combine with tetanus toxin—this might take place with or without resulting symptoms. It is, moreover, evident that the molecules in the cells which unite with toxin may, when set free, act as antitoxin by neutralising the toxin and thus preventing its combination with the cells. This will be referred to below in connection with the theory by which Ehrlich accounted for the development of antibodies (p. 246).

Site of Origin of Antibodies.—The experimental investigation of the site of origin of antibodies has yielded comparatively few facts, but their local production has been demonstrated in several instances. In the case of rabbits undergoing immunisation with killed *V. choleræ*, Pfeiffer and Marx found a greater concentration of bactericidal antibody in the spleen, bone marrow, and lymph glands than in the blood, and similar observations have been made with regard to agglutinins. After the injection of foreign protein into the anterior chamber of the eye, precipitin has been detected in the aqueous humor of that eye before it was present in the blood (Dungern). Recently, in view of the phagocytic capacity of cells of the reticulo-endothelial system, it has been supposed that they produce the antibodies. It appears likely that these cells are concerned in the production of antagonistic and protective substances, but no quite conclusive evidence for this has been obtained. Attempts to demonstrate interference with antibody formation by " blockade " of the reticulo-endothelial system have led to inconclusive results. Buttle (1934), has shown in the case of rabbits immunised with diphtheria toxoid that neither the blood, liver and spleen, nor the skin is predominantly responsible for the production of antitoxin. Hæmolytic immune-body is stated to have been formed after a few days in tissue cultures of bone marrow and lymph gland, and bactericidal immune-body in

that of splenic tissue, to which the respective antigens had been added. It is to be noted, however, that antibodies have never been demonstrated in extracts from leucocytes or other cells, and a similar statement holds with regard to complement.

Of the *chemical nature of antibodies* little is known. From filtration experiments Brodie, and Martin and Cherry, deduced that the antitoxins were large molecules. It had previously been found that the precipitate of globulins obtained by adding appropriate concentrations of magnesium or ammonium sulphate to anti-diphtheria serum, anti-tetanus serum, or the milk of immunised animals, contained practically all the antitoxin. Further, it was determined by Ide and Lemaire, Pick, and others that the antitoxin was not uniformly distributed amongst the various globulin fractions which may be obtained from sera, but that it was largely restricted to a particular component—the pseudo-globulin in the case of diphtheria antitoxin from the horse. Hiss and Atkinson, and also Ledingham, found that the percentage amount of globulin precipitate from the serum of the horse increased after it was treated in the usual way for the production of antitoxin; this increase, however, does not appear to be an essential accompaniment of antibody formation.

Similar results have been obtained with regard to the other classes of antibodies. They are in general found along with the globulins, though the fraction with which they are chiefly associated varies in different cases; and the possibility must be considered that they may merely be adsorbed by the globulins. This association, however, affords a valuable basis for practical methods of concentrating and purifying antitoxin and other antibodies. Another procedure which has been employed in attempts to isolate antibodies consists in combining a particulate antigen, *e.g.* bacteria, with the corresponding antibody, or in the case of an antigen in solution, such as a toxin, obtaining a precipitate with the antitoxin. Material remaining in solution is then removed by centrifuging, and the residue is washed and finally dissociated by altering the reaction or the salt content of the medium or by electrical dialysis. From the evidence established, it appears likely that antibodies are really globulins, but this cannot be definitely stated, as they have not yet been obtained in pure form. It may be mentioned in this connection that Huntoon and his co-workers have separated antibodies from their combination with pneumococci, and have found that they do not correspond in their reactions with serum proteins; for example, they are not affected by trypsin, and are not precipitated, like globulins, by saline solutions.

Regarding the nature of the complex which is formed by antigen and antibody, little is known. As has been seen, provided the action of complement is excluded the combination is almost always a reversible one. It has been supposed that the antibody globulin forms a layer covering the antigen and that a regular arrangement of the molecules takes place on the analogy of that which is supposed to occur at surfaces of separation under the influence of polar forces, as in a condensed film of palmitic acid floating on water, where the carboxyl groups are all orientated toward the water and the hydrocarbon chains toward the air. Owing to the small amount of antiserum which may be effective in bringing about an immunity reaction, *e.g.* agglutination, it is possible that the antibody does not completely coat the surface of the antigens, but simply becomes attached at particular places. In this way the antibody would serve to link the molecules of the antigen together and so produce a lattice (Marrack).

Antitoxin, when present in the serum, leaves the body in various secretions, and in these it has been found, though in much less concentration than in the blood. It is present in the milk, and a certain degree of immunity can be conferred on new-born animals by feeding them with such milk, as has been shown by Ehrlich, Klemperer, and others. Bulloch also found in the case of hæmolytic sera that the antibody is transmitted from the mother to the offspring in the milk. In the case of goats and cattle it is the colostrum which is specially rich in antibodies. In certain other animals, such as the guinea-pig and rabbit, the actively or passively immunised mother transmits antibodies to the fœtus through the placenta. Klemperer found traces of antitoxin in the yolk of eggs of hens whose serum contained antitoxin.

As met with in untreated specimens of antisera, antibodies are damaged or destroyed by agencies which denaturate proteins. Thus, while they may resist heating at 55° to 65° C. for several hours, higher temperatures are rapidly destructive ; and when antisera are kept at 16° to 37° C. for long periods antibodies gradually disappear. On the other hand, desiccation or freezing preserves them practically indefinitely. The gradual deterioration of antibodies when sera are stored under ordinary conditions is of practical importance in connection with their therapeutic use.

Mechanism of Antibody Formation : Ehrlich's Side-Chain Theory.—At present this theory is the only one which even attempts to explain the cardinal facts of antibody formation. It may be said to be an application of views regarding the assimilation of nutrition by cells. A molecule of protoplasm may be

regarded as composed of a central atom group or functional centre with a large number of side-chains, *i.e.* atom-groups with combining affinity for nutritive substances of complex constitution. It is by means of these latter that the living molecule is built up in the process of nutrition, and hence the name *receptors* given to the combining apparatus by Erhlich is appropriate. In considering the application of this idea to the facts of acquired immunity, it must be kept in view that all the substances to which antibodies have been obtained are undoubtedly of complex chemical constitution, and that in apparently every case the antibody enters into combination with its corresponding antigen. The dual constitution of toxins and kindred substances, as already described (p. 198), is also of importance in this connection. Now, to take the case of toxins, when these are introduced into the system they are fixed, like nutritive substances, by their haptophore groups to the receptors of the cell protoplasm, but they are unsuitable for purposes of nutrition. If they are in sufficiently large amount, the toxophore part of the toxin molecule produces that disturbance of the protoplasm which is shown by symptoms of poisoning. If, however, they are in smaller dose, as in the early stages of immunisation, fixation to the protoplasm occurs in the same way; and as the combination of receptors with toxin is supposed to be of firm nature, the receptors are lost for the purposes of the cell, and the combination R-T (receptor + toxin) is shed off into the blood. The receptors thus lost become replaced by new ones, and when additional toxin molecules are introduced, these new receptors are used up in the same manner as before. As a result of this repeated loss, the regeneration of the receptors becomes an over-regeneration, and the receptors formed in excess, instead of all remaining attached to the protoplasm of the cells, *i.e.* "sessile," now appear in the free condition in the blood stream and then constitute antitoxin molecules. There are thus three factors in the process, namely (1) fixation of toxin, (2) over-production of receptors, (3) setting free of receptors produced in excess. Accordingly, these receptors which, when forming part of the cell protoplasm, anchor the tox n to the cell and thus are essential to the occurrence of toxic phenomena, in the free condition unite with the toxin, and thus prevent the toxin from combining with the cells and exerting a pathogenic action. Ehrlich did not state what cells are specially concerned in the production of antibodies, but from what has been stated it is manifest that any cell which fixes toxin is potentially a source of antitoxin Cells to whose disturbance, resulting from the fixation of toxin,

characteristic symptoms of poisoning are due, ought thus to be sources of antitoxin, *e.g.* cells of the nervous system in the case of tetanus, though the cells not seriously affected by toxin fixation may act in the same way.

It will be seen that Ehrlich's theory accords well with many of the known facts of active and passive immunity, and up to a point it affords an explanation of the multiplicity of antibodies. For, if we take the case of antitoxin, we see that this depends upon the combining affinity of the toxin for certain of the cells of the body, and this again is referred back to the complicated constitution of living protoplasm. It is to be noted, however, that it does not explain active immunity apart from the presence of antibodies in the serum. For example, an animal may be able to withstand a much larger amount of toxin than could be neutralised by the total amount of antitoxin in its serum, or may possess immunity when antibodies have disappeared from the blood. This might theoretically be explained by supposing a special looseness of the cell receptors so that the toxin-receptor combination became readily cast off. The question, however, arises whether there may not be really an increased resistance of the cells to the toxic action—a genuine tissue immunity. An observation made by Meyer and Ransom (*vide* p. 637) is also difficult of explanation, according to the view that antitoxin is formed by the cells with which the toxin combines and on which it acts. They found that in an animal actively immunised against tetanus and with antitoxin beginning to appear in its blood, the injection of a single M.L.D. of tetanus toxin into a peripheral nerve brought about tetanus with a fatal result. On the other hand, the injection of antitoxin into the sciatic nerve above the point of injection of toxin prevented the latter from reaching the cells of the cord. One can scarcely imagine an explanation of these facts if antitoxin molecules were in process of being shed off by the cells of the nervous system. Further, when the serum of an animal contains a large amount of antitoxin, how does the additional toxin injected reach the cells in order to influence them as we know it does? This also is difficult to understand, unless the toxin has a greater affinity for the receptors in the cells than for the free receptors (antitoxin) in the serum. A further difficulty which attends the theory is that antibodies cannot be recovered to any marked degree from the blood-free tissues of immunised animals; on the other hand, the existence of sessile receptors is supported by observations in connection with anaphylaxis (p. 261). One point which Ehrlich's theory does not take into account is that

antigens, in respect of their foreign specificity, differ from the natural food-stuffs of cells, since such materials before absorption are normally deprived of specificity by the ferments in the alimentary tract. Accordingly, antibody formation represents a biological reaction on the part of the tissues when they come into contact with substances possessing such foreign specificity. A recent theory which embodies the essentials of Ehrlich's view, is that the injected antigen (or its derivative) on reaching the sites where globulin is being formed in the tissues, leads to some distortion of the products which is characteristic for the particular antigen (Breinl and Haurowitz)

Non-Specific Factors influencing Antibody Formation.—As stated above, it is clear that antibodies are formed by the cells of the body, and it has now been shown that their formation can be influenced by factors other than the injection of antigens. For example, it has been found that if the blood of an animal containing antibodies is removed and replaced by the blood of a normal animal, the antibodies are rapidly restored. And further, repeated small bleedings may raise the content of the blood in antibodies above its former level. Acting on the supposition that antitoxin formation is allied to a process of secretion, Salomonsen and Madsen tested the effect of pilocarpine and found that it had the property of producing a marked rise in the amount of antitoxin present in the blood of an animal. Walbum and others have also tested the effects of various metallic salts and have found that many have a similar property of stimulating the formation of antibodies. These were tested on immunised animals after the antibody content had fallen to a steady level. Salts of different metals vary much in their action, but they found that amongst the metals of the iron group manganese chloride has the greatest effect, this salt sometimes rapidly raising the amount of antibodies almost to the previous maximum. The most efficient salt of all, however, was beryllium chloride. Mackie has shown that the normal antibody of the rabbit for sheep's corpuscles is increased by extensive bleedings and also by the administration of metallic salts (manganous chloride and beryllium chloride), colloidal manganese, salvarsan, etc. The explanation of such non-specific stimulation is quite obscure. These non-specific factors are irregular in their action, however. Further aspects of non-specific formation of antibodies are discussed under natural immunity (p. 251).

Therapeutic Use of Antisera.[1]—The chief human diseases

[1] The Therapeutic Substances Act, 1925, applies to the manufacture for sale of antisera in the same way as to vaccines (p. 158). The Therapeutic Substances Regulations, 1931 and 1935, prescribe conditions with respect to which commercial therapeutic antisera must conform as regards their mode of preparation, sterility, freedom from abnormal toxicity, and content in solid matter; also in the case of diphtheria and tetanus antitoxins, gas gangrene antitoxins (perfringens, œdematiens and vibrion septique), staphylococcus antitoxin, anti-dysentery serum (Shiga and others), and anti-pneumococcus serum (types I. and II.), the methods of standardising and the potency in relation to volume of serum or weight of dry products are prescribed.

treated by antitoxic sera are diphtheria, tetanus, botulism, scarlet fever and other streptococcal toxæmias, staphylococcus infection, gas gangrene, snake-bite, and scorpion sting; and by antibacterial sera, streptococcus infections, cerebro-spinal fever, pneumonia, dysentery, and plague. The methods of application in bacterial infections and the general results will be dealt with under individual diseases. When large doses have to be given, concentrated preparations of antisera are commonly employed, which consist of the globulin fraction to which the antibodies are attached. Antibacterial sera have not, on the whole, proved so efficacious therapeutic agents as antitoxic sera.

As has been shown above, antibacterial sera require for their bactericidal action a sufficiency of complement, and as this becomes lost when a serum is kept, the unsatisfactory results with this class of sera may be due to a deficiency of complement. Or it may be, as Ehrlich suggested, that the complement naturally existing in human serum does not suit the immune-body in the antiserum—that is, is not taken up through the medium of the latter and brought into combination with the bacterium. And there is the further possibility that even though the complement should be taken up, the toxic action of the latter may not be sufficiently active towards the bacterium to effect its death. In both cases it will appear that an extracellular bactericidal action cannot be produced by the particular immune-body in association with the complement of the animal in question. There is no doubt that this question of differences in complements is one of high importance, and that both combining affinity and toxic action of complements must be considered in each case.

In such diseases as cerebro-spinal fever and pneumonia the opsonic mechanism of the infected individual may play a part in successful resistance. The favourable effects following treatment with antisera may thus, in some cases, depend on an augmentation of the opsonic powers of the body.

In addition, there is the question of adequate dosage of the antibody; it has been found experimentally that an antibacterial serum in amounts short of the curative dose may produce little or no beneficial effect. Further, beyond a certain degree of infection no amount of serum will protect.

Infection Immunity: Tissue Immunity

In some infections manifestations of immunity are met with which cannot be explained satisfactorily by the mechanisms so

far considered. For instance, after infection with *Tr. pallidum* the skin soon ceases to respond to a further inoculation with the spirochætes at a fresh site by the development of another chancre. This resistance to " superinfection " persists so long as the body still harbours the spirochætes, but if these are destroyed as the result of administering chemotherapeutic agents, the susceptibility of the skin to reinoculation soon returns. It appears therefore that this form of immunity lasts only so long as the infection persists. Accordingly, this condition has been called " infection immunity." It must be noted, however, that such immunity is only of a very limited nature, since the refractory state of the skin is not necessarily accompanied by absence of lesions in other parts of the body. In other infections, especially those caused by protozoa, a similar state is met with, *e.g.* in malaria, where during latency of the disease attempts at superinfection fail. In malaria also, we have a striking example of an infection in which the occurrence of latency or cure cannot be accounted for by the appearance in the blood of any species of antibody. It must be concluded that the immunity is due to a heightened capacity of the tissues, especially the reticulo-endothelial system, to destroy the parasites, possibly accompanied by the development of some form of tolerance to those which persist ; to this the term " tissue immunity " has been applied. Regarding the nature of these forms of immunity little is known, however.

Natural Immunity

We have placed the consideration of this subject after that of acquired immunity, as the latter supplies facts which indicate in what direction an explanation of the former may be looked for. There may be said to be two main facts with regard to natural immunity. The first is, that there are large numbers of bacteria —the so-called non-pathogenic organisms—which are practically incapable, unless perhaps in very large doses, of producing pathogenic effects in any animal ; when these are introduced into the body they rapidly die out. This fact, accordingly, shows that the animal tissues generally have a remarkable power of destroying living bacteria. The second fact is, that there are other bacteria which are very virulent to some species of animals, whilst they are almost harmless to other species ; the anthrax bacillus may be taken as an example. Now it is manifest that natural immunity against such an organism might be due to a special power possessed by an animal of destroying the organisms

when introduced into its tissues. It might also possibly be due to an insusceptibility to, or power of neutralising, the toxins of the organism; for the study of the various diseases shows that the toxins (in the widest sense) are the agents by which morbid changes are produced, and that toxin-formation is a property common to all pathogenic bacteria. As a matter of fact, however, natural immunity is, in most cases, one against *infection*, *i.e.* consists in a power possessed by the animal body of destroying the living bacteria when introduced into its tissues: such a power may exist though the animal is still susceptible to the separated toxins. We shall now look at these two factors separately.

1. Variations in Natural Bactericidal Powers.—The fundamental fact here is that a given bacterium may be rapidly destroyed in one animal, whereas in another it may rapidly multiply and produce morbid effects. The special powers of destroying organisms in natural immunity have been ascribed to (*a*) phagocytosis, and (*b*) the action of the serum.

(*a*) *Phagocytosis.*—The part played by the leucocytes and by the phagocytes of the reticulo-endothelial system, which are accumulated especially in the spleen, liver, bone marrow and lymph glands, in removing organisms from the blood and lymph, has already been dealt with (pp. 183, 244). The chief factors with regard to phagocytosis have also been given above. The bacteria in a naturally immune animal, for example, the anthrax bacillus in the tissues of the white rat, are undoubtedly taken up in large numbers and destroyed by the phagocytes, whereas in a susceptible animal this only occurs to a small extent; and Metchnikoff showed that in the latter case they are living, and are still virulent when tested in a susceptible animal. Variations in phagocytic activity are found to correspond more or less closely with the degree of immunity present, but are probably in themselves capable of explanation. The fundamental observations of Wright and Douglas (p. 229) show that an essential in phagocytosis is the labile opsonin of normal serum, which has combining affinities for a great many organisms, as already stated. In many instances, however, natural antibodies are concerned in the opsonic action, as in the case of the protective effect of normal fowls' serum against pneumococci in mice (Bull and McKee). But the all-important fact is that whether phagocytosis occurs or not appears to depend upon certain bodies in the serum. As yet, we cannot say whether the phagocytosis in a given serum, observed according to the opsonic technique, always runs parallel with phagocytosis in the

tissues of the animal from which the serum has been taken, but it has been found in many instances that the results *in vivo* and *in vitro* closely correspond. In the non-occurrence of phagocytosis seen when an organism acquires increased virulence, two main factors are concerned. On the one hand, the insensitiveness of a bacterium to opsonic action may be associated with recognisable changes in the bacterium, notably by the formation of a capsule, and this has been demonstrated in certain instances. On the other hand, the virulent condition is sometimes due to the production by the bacterium of substances such as aggressins (p. 192) which have a harmful effect on the phagocytes. Ingestion is, however, only the first stage in the process ; intracellular destruction is the second, and is of equal importance. The mechanism by which the phagocytes of the reticulo-endothelial system destroy ingested bacteria is still obscure, however. What may be called intracellular bactericidal action probably varies in the case of phagocytes of different animals, and, further, bacteria sometimes survive the cells which have ingested them, the latter undergoing necrosis and disintegration. In other instances, the organisms do not appear to suffer from their intracellular position ; an example of this is afforded in the case of the gonococcus. Variations in the susceptibility of organisms to the intracellular bactericidins is thus another important factor in natural immunity, though these in their turn cannot be explained.

(*b*) *Bactericidal Action of the Serum.*—When it had been shown that normal serum possessed bactericidal powers against different organisms, the question naturally arose as to whether this bactericidal power varied in different animals in proportion to the natural immunity enjoyed by them. The earlier experiments of Behring appeared to give grounds for the belief that this was the case. He found, for example, that the serum of the white rat, which has a remarkable immunity to anthrax, had greater bactericidal powers than that of other animals investigated. Further investigation, however, has shown that this is not an example of a general law, and that the bactericidal action of the serum does not vary *pari passu* with the degree of immunity. In some cases non-pathogenic and also attenuated pathogenic bacteria can be seen to undergo rapid solution and disappear when placed in a drop of normal serum. Recently Abdoosh has found that normal human serum is not bactericidal toward the gonococcus, while this organism is readily killed by the sera of many other mammals which are insusceptible to the infection. Again Schütze and others have shown that strains

of mice which are markedly resistant to Salmonella infection possess more natural antibody to these organisms than do susceptible strains (p. 535). In the case of many pathogenic organisms, however, the serum has no direct bactericidal effect at all. The bactericidal action of the serum was specially studied by Nuttall, and later by Buchner and Hankin, who believed that the serum owed its power to certain substances in it, derived from the spleen, lymphatic glands, thymus, and other tissues rich in leucocytes. To these substances Buchner gave the name of *alexins*; as already explained, they correspond with Metchnikoff's cytases and Ehrlich's complements, usually in association with natural immune-bodies. As already stated, complement does not increase in amount during immunisation, and we may add that there is no satisfactory evidence that its source is the leucocytes or that it is set free from them only after the blood is shed, as has been maintained by various French writers. In the case of the hæmolytic action of a normal serum, it has been shown in many instances that, in addition to complement, a natural immune-body is also concerned (p. 227), and this would appear to be the rule; the process being analogous to what is seen in the case of an artificially developed hæmolytic serum. In many instances an analogous condition obtains also in the case of normal bactericidal sera. For example, ox's serum, heated at 55° C., contains a natural immune-body to *V. choleræ* which can be activated by the addition of complement so as to produce a bactericidal action, though complement is by itself without any such effect. Mackie and Finkelstein have shown that a normal serum may contain a number of such natural immune-bodies which are specific in character. On the other hand, Gordon attributes the bactericidal action of normal serum, in the examples which he studied, to the joint action of complement and a non-specific heat-stable intermediary factor. Variations in bactericidal power of the serum due to complement as tested *in vitro*, however, do not explain the presence or absence of natural immunity against a living bacterium. In some cases, for example, it has been found to be considerable, while the organisms flourish in the body and the animal has no immunity.

In addition to that exerted through the medium of complement, bactericidal action has been shown to be possessed by certain substances extracted by physical or chemical means from leucocytes—*leukins*, and also from platelets—*plakins*. (It has been shown that the blood-platelets very rapidly adhere to various organisms and foreign cells which have been injected

into the circulation, and these show evidence of undergoing lysis in certain cases (Taniguchi).) Bactericidins of this class are, in contrast to complement, not destroyed at 55° to 60° C. for half an hour, and are little affected by salt concentration. Moreover, their action is a direct one, immune-body not being concerned, and is efficient on different organisms from those killed by complement. Their activity, however, is found to vary greatly in the case of different animals. It is generally held that bodies of the leukin class are set free only on destruction of the cells, though some writers consider that this may occur also by a process of secretion. Such substances apparently play an important part especially in the intracellular killing of organisms, and variations in resistance may sometimes depend on them.

Another type of bactericidal substance exists normally in serum and acts especially on *B. anthracis* and certain other Gram-positive organisms, which Pettersson has described as *β-lysin.* This differs from complement in many respects, being especially more thermostable. *Lysozyme,* found by Fleming in various secretions, especially tears, and in tissues, causes rapid solution of some saprophytic cocci. The properties of all these bodies undergo no increase as the result of immunisation.

Important evidence regarding natural immunity to certain infections may be obtained by investigating the *combined action of phagocytosis and serum* on bacteria. In this way the summation effects of extra- and intra-cellular antibacterial mechanisms are obtained. This may be done by exposing the organisms to the action of whole blood. By this method a definite correlation has been found to exist between the bactericidal power of the blood of rats for *B. pestis* and immunity to infection with that organism (Malone, Avari and Naidu). Similarly, Bull and Tao have shown by using citrated whole blood inoculated with varying doses of pneumococci, that in the case of an insusceptible species (chicken) the inoculum required to yield growth of the organisms is over one million times greater than is required with the blood of a susceptible species (rabbit). Instead of whole blood, mixtures of serum and leucocytes may be allowed to act on the organisms.

2. Variations in Natural Susceptibility to Toxins.—We must here start with the fundamental fact, incapable of explanation, that toxicity is a relative thing, or, in other words, that different animals have different degrees of resistance or non-susceptibility to toxic bodies. In every case a certain dose must be reached before effects can be observed, and up to that point the animal has resistance. This natural resistance is

found to present very remarkable degrees of variation in different animals. The great resistance of the common fowl to the toxin of the tetanus bacillus may be here mentioned (*vide* p. 633), and large amounts of this poison can be injected into the scorpion without producing any effects whatever. The high resistance of the rat to diphtheria toxin is another striking example. This variation in resistance to toxins applies also to those which produce local effects, as well as to those which cause symptoms of general poisoning. Instances of this are furnished, for example, by the vegetable poisons ricin and abrin, by the snake poisons, and by bacterial toxins such as that of diphtheria. The insusceptibility of the new-born to certain toxins has already been referred to (p. 173). According to Ehrlich's view of the constitution of toxins, such insusceptibility might be due to the want of combining affinity between the tissue cells and the haptophore group of the toxin; or, on the other hand, supposing this affinity to exist, it might be due to an innate non-susceptibility to the action of the toxophore group. Investigations have been made in order to determine the combining affinity of the nervous system of the fowl with tetanus toxin, as compared with that obtaining in a susceptible animal, but the results have been somewhat contradictory. Accordingly, a general statement on this point cannot at present be made, though in all probability variations in the susceptibility to the toxophore group will be found to play a very important part. It was shown by Muir and Browning by means of hæmolytic tests that the toxic activity of complement, after it has been fixed to the corpuscles, varies very much; in some instances an amount of complement which would rapidly produce complete lysis of one kind of corpuscle, may have practically no effect on another, even though it enters into combination. These results are of importance in demonstrating how the corresponding cells of different animals may vary in sensitiveness to toxic action.

In certain diseases, *e.g.* diphtheria and scarlet fever in this country, susceptibility is greatest in childhood and diminishes with age. The increase in resistance is due chiefly to the appearance in the serum of antitoxins for the toxins of the causal organisms, although the individuals have never suffered from clinically recognisable attacks. According to one view the explanation of the occurrence of these antibodies in the serum is that the corresponding organisms are widely prevalent in the community and therefore in the course of time most persons acquire a series of "subclinical" infections which lead to their development. Observations on the behaviour of diphtheria in

a semi-isolated community strongly support this view (Dudley). On the other hand, from a study of the distribution of natural lysins and agglutinins for sheep and rabbit red cells respectively in the blood of children at different ages, Friedberger has argued that the presence of antibodies is associated with maturation of the tissues. This explanation may not be conclusive, since those animals are so widely consumed as food, but the natural lysin for sheep's corpuscles in rabbit's serum behaves similarly. Gibson has made a like observation in regard to natural agglutinins, where infection with the organisms could be excluded practically. Also, certain antibodies are formed under circumstances which exclude a reaction to a specific antigen. Such are the hæmolytic immune-body for ox's red corpuscles in normal guinea-pig's serum ; also the agglutinin for *B. mallei* in the serum of horses in Great Britain although glanders does not exist there (Lovell). Further, the different anatomical distribution of the same infection at varying ages, *e.g.* tuberculosis, and the predilection of certain infections for particular age groups, indicate clearly that maturity of the tissues plays an important part in determining the incidence of infections. Again, the tissues may naturally possess defensive mechanisms which are not explicable on the analogy of an acquired immunity. For instance, Flexner found that certain monkeys were refractory to inoculation with poliomyelitis virus placed on the nasal mucous membrane, but these animals possessed no special resistance to intracerebral inoculation and their serum had no action in neutralising the infectivity of the virus.

If we review the whole subject of natural immunity we see that variations in natural immunity depend in the main upon variations in phagocytosis and consequent intracellular bactericidal action, as already explained. In the case of different organisms the result depends on variations in their susceptibility to the bactericidins of the fluids and cells and on variations in the susceptibility of the tissues to toxins (in the widest sense) formed by the bacteria.

Mass or Herd Immunity

Hitherto the reactions of the individual have been taken into account almost exclusively in regard to infection and immunity. But it is necessary to consider the behaviour of aggregates of individuals as they exist in human communities or herds of animals. Until recently information about infective diseases among masses was almost purely descriptive. Certain of these

diseases were known to become epidemic at intervals, the numbers of cases at such times being large as compared with that in the intervals. A disease with very regular periodic epidemics is measles in this country; epidemics of influenza and poliomyelitis, on the other hand, are highly irregular in their occurrence. From the observations of Farr and others, it appears that epidemics generally follow a regular course as regards their onset, acme, and decline; also the subsidence of an epidemic occurs long before the number of susceptibles is exhausted. It seems likely therefore that the onset and progress of an epidemic are due to some initial, almost explosive increase in infectivity or pathogenicity of the causal agent, which later diminishes again (Brownlee). Of course, alterations in the susceptibility of the population might also be responsible, but it is difficult to conceive that a change of this nature on the part of the hosts can be world-wide, as would have to be the case in order to explain a pandemic like that of influenza in 1918. So far, however, it has not been possible to investigate the causal agent of a natural epidemic from this point of view, although cyclical changes in virulence of the virus of poliomyelitis under conditions of experimental transmission have been established (p. 751). On the other hand, the regular recurrence of epidemics of measles at about two-yearly intervals may well depend on the accumulation of highly susceptible children born in the intervals between epidemics. In the case of endemic infections such as tuberculosis, the alterations in prevalence and severity take place over much longer periods. The circumstances of a natural population are so complex as to render a detailed analysis of the conditions almost impossible. Factors affecting the hosts, such as emigration and immigration, varying susceptibility due to sex and age, occupation, nutrition, etc., must all be borne in mind; also accidents leading to excessive exposure must be regarded, *e.g.* where a defective drain from an isolation hospital has permitted sudden access of infective matter from a case of enteric to the water supply. All these points obviously influence the spread of infections. It must also be borne in mind that the practice of isolation has had little effect on the spread of epidemic diseases, chiefly, no doubt, on account of the fact that cases are clinically recognisable only at certain stages and also that a proportion cannot be recognised at any time in their course.

In order to study the problems under somewhat simpler conditions the behaviour of infective diseases in semi-closed communities has recently been investigated, especially in the case of diphtheria, by Dudley (p. 256); also epidemics have been

set going in collections of small animals such as mice, and the influence of adding fresh susceptible individuals at different rates or of adding immunised animals has been studied by Greenwood and Topley in this country, and at the Rockefeller Institute in New York. The procedure adopted was to infect adult mice by feeding, *e.g.* with mouse typhoid or pasteurella bacilli, and then to introduce similar uninfected animals into the infected community, the rate and number of introductions being varied in different experiments. When normal mice were introduced it was found that practically all became infected eventually and that soon deaths among these occurred, which after a time gradually diminished in number until a point was reached at which the survivors had a somewhat better expectation of life than more recent immigrants. The weeding out of susceptibles probably contributes in part to this resistance, but it appears to be due chiefly to a process of immunisation, especially since it tends to be specific for the organism concerned. In the case of ectromelia, a virus infection in mice, a fairly high degree of immunity develops eventually.

The next question is the influence upon the fatality, produced by adding immunised animals to the infected herd. With mouse typhoid vaccines a distinct but not high degree of protection was conferred in the sense that the treated animals survived introduction into the infected community longer than did normal ones. But a large proportion became infected nevertheless. The protection conferred by a specific vaccine was much greater in the case of ectromelia, at least in the early periods after entry to the herd. Once a herd has been infected by mouse typhoid, however, the introduction of immunised animals only, does not lead to disappearance of the infection. The introduction of bacteriophage has also been ineffective in this respect.

The experiments did not throw light on the hypothesis that natural epidemics start from the development of an " epidemic " strain of the organism, although variations in infectivity were found to occur in the organisms isolated during the course of experimental epidemics. But they serve to demonstrate the distinction between the capacity of a strain to spread naturally from host to host and its capacity to cause severe illness in infected individuals. It was found that an organism might infect a large proportion of animals exposed to it and nevertheless cause only small fatality. Accordingly it appears likely that a strain which is capable of causing an epidemic must combine both the properties of infectivity and pathogenicity.

Supersensitiveness, Anaphylaxis and Allergy

The term *supersensitiveness* is applied to states where, as compared with the normal, there is increased susceptibility or reactivity to substances introduced into the body, either parenterally or through the alimentary canal. The condition may be manifested by either local or general disturbances, and it may be natural or acquired. It has long been recognised that the ingestion of some substances, *e.g.* shell-fish, strawberries, etc., by certain individuals, may be followed by constitutional disturbances, and also that the injection of a minute amount of foreign serum may give rise to such disturbances. A particular individual, however, is likely to be supersensitive to only one of these various substances. Such supersensitiveness has been termed *natural*, but it is doubtful whether in most cases it does not result from previous contact with the substance; accordingly, it may come into the same category as the next examples. The increased reactivity of patients or animals suffering from an infection to the products of the infecting organism is a noteworthy example of *acquired* supersensitiveness. Thus we have the tuberculin and mallein reactions, and here both local and general effects are met with. Jenner probably described the first instance of allergy in infective disease when he noted that in an individual who had suffered from cowpox and therefore was immune to smallpox, inoculation of the skin with variolous matter led to an inflammatory reaction more rapidly than did a similar inoculation in a susceptible person.

It has come to be recognised that there is a special form of supersensitiveness which is induced by the injection of substances with antigenic properties, the toxic phenomena becoming manifest only on the re-injection of the antigen after a suitable interval of time. In this form the disturbances are now known to be the result of the interaction of antibody and antigen. Thus, while the phenomena in their characters and results present a striking contrast to the state of immunity, they are in their mode of production closely related to the latter condition. To this form of supersensitiveness the term "anaphylaxis" is generally applied, and it is advisable to restrict its use in this way. The substances which have been found to have the property of calling forth the anaphylactic state—anaphylactogens—are of various kinds, including bacteria and their toxins, animal poisons, and a great many foreign proteins, *e.g.* those of serum, milk, egg albumin, etc. They are apparently all of protein nature, and they are probably coextensive with the

substances which act as antigens in immunity reactions. It is important to note that many of the substances which act as anaphylactogens are on their first injection perfectly harmless. In order to produce anaphylactic shock in sensitised animals it is not always essential to inject the complete antigen, since the corresponding haptens may be effective. Thus, in animals sensitised with antisera to *B. lactis aerogenes* the injection of the specific carbohydrate substance produced anaphylactic shock (Tomcsik and Kurotchkin). Recently Landsteiner and Jacobs have shown that supersensitiveness (both local and general) can be produced by chemical substances, *e.g.* salvarsan; and anaphylactic shock may follow intravenous injection of this drug in a supersensitive person. Presumably the drug combines with some protein in the tissues, and this complex acts as the anaphylactogen.

Many of the examples of supersensitiveness, *e.g.* the tuberculin and allied reactions, have not yet been proved to depend on the interaction of antigen and antibody—that is, to be of anaphylactic nature. To such supersensitiveness the term " allergy " (altered reactivity) may be applied, as suggested by Coca. To the analogous conditions of hay fever, asthma, susceptibility to certain articles of diet, etc., he has given the name of " atopy " (strange disease). The group is, however, not a homogeneous one, and, in most instances, the mode of production is obscure; the nature of each must be investigated separately.

Anaphylaxis.—At a comparatively early date it was found, in the case of diphtheria and tetanus toxins, that, especially in small animals, the injection of a minute dose followed by another at a suitable interval might be attended by serious results; and that this was not an example of accumulative action, was shown by the fact that the sum of the doses might amount to only a fraction of a lethal dose. Richet investigated a similar phenomenon in the case of a toxic substance obtained from the tentacles of actiniæ, to which, from its action, he gave the name of " congestin." He found that a certain time-interval between the injections was necessary; that after the second injection the symptoms occurred with remarkable suddenness, and that they appeared to be practically independent of the size of the first dose. He applied the term *anaphylaxis* to the supersensitive condition, and this has passed into general use; he found also that the condition lasted several weeks at least. Arthus found that after repeated injections of horse serum in rabbits, a stage was reached at which an additional subcutaneous injection produced marked œdema and even necrosis, while an intravenous

injection, harmless to an untreated animal, brought about a fatal result. The period of active research on the subject, however, may be said to date from the discovery of what is now known as the "phenomenon of Theobald Smith." This observer found that guinea-pigs which had been treated with a neutral mixture of diphtheria toxin and antitoxin might, after a certain interval of time, succumb on being injected with a quantity of normal horse serum. It was afterwards shown—especially by the researches of Otto and of Rosenau and Anderson—that the sensitising agent had really nothing to do with the toxin or antitoxin, but was due to the constituents normally present in the serum.

Phenomena of Active Anaphylaxis.—After this brief review we may consider some of the phenomena of anaphylaxis. In its study various sera and proteins from other sources have been employed, and the guinea-pig is the most suitable test animal on account both of the minute amounts which render it anaphylactic and of the certainty with which severe symptoms can be produced. The rabbit has also been used, but its relative susceptibility is less than a hundredth of that of the guinea-pig. In the case of mice or rats, it is difficult if not impossible to bring about serum anaphylaxis. There is, first of all, the *sensitising* injection; a guinea-pig is injected subcutaneously with a minute quantity, *e.g.* 0·01 c.c of serum, though even 0·000001 c.c. has been found sufficient in certain instances; other methods of injection may also be employed. After a certain number of days, usually ten as a minimum, anaphylaxis has been established, and the test for this is usually made by injecting by the intravenous route 0·5 c.c. of the same serum or even less, or intraperitoneally 5 c.c.—the *assaulting* dose. The former method brings about the result more rapidly. Subcutaneous injection is less effective and less certain. In the anaphylactic animal, especially after an intravenous injection of serum, acute shock occurs, which causes death usually within a few minutes. Restlessness and hyperalgesia are followed by evidence of collapse, urine and fæces are passed, the heart's action becomes weak and the respiration embarrassed: in fatal cases respiration stops before the heart's action ceases. In the more protracted form of shock following an intraperitoneal or subcutaneous injection the animal's temperature falls markedly and it becomes comatose. It is to be noted that the minimum amount of serum necessary to bring about the symptoms of fatal anaphylactic shock is much greater (it may be one thousand times) than the original sensitising dose; and that while anaphylaxis is not fully established till about the tenth

day, it occurs gradually—not by crisis—as can be shown by disturbance of the temperature on re-injection of serum at a much earlier period. Anaphylaxis has the character of specificity, apparently within corresponding limits to immunity (p. 210)—that is, it is manifested only on the re-injection of the same protein substance as that used in the first instance.

Passive Anaphylaxis.—There is also a passive anaphylaxis, as is shown by the fact that if a certain amount of the serum of an anaphylactic guinea-pig be injected into a normal one, the latter becomes anaphylactic, so that the characteristic symptoms appear in it when the test amount of antigen (anaphylactogen) is injected. In the guinea-pig shock is produced with greatest certainty if an interval of some hours has elapsed between the injections (Otto); if the two injections are made at the same time there is frequently no result, but this is not invariably the case (Dean and others). This fact is of importance in connection with the nature and mode of causation of anaphylaxis. In the rabbit, however, the symptoms appear almost at once if the two injections are given practically at the same time. The serum need not be derived from the same species as the animal which is to be rendered passively anaphylactic. Passive anaphylaxis usually disappears after a few weeks at longest, whereas active anaphylaxis has been observed after more than two years; here also there is an analogy between anaphylaxis and immunity. Another interesting observation has been made, namely, that the young of anaphylactic mothers may also be anaphylactic, and the condition may last for some time after birth.

Kellet has shown recently that anaphylactic shock may be produced by injecting guinea-pigs with foreign serum followed after an interval by a large dose of the corresponding antiserum from the rabbit, both injections being given intravenously; to this phenomenon the term *reversed passive anaphylaxis* has been applied. According to Zinsser and Enders, however, only exceptional animals behave in this way and there need be practically no interval between the two injections.

Anti-anaphylaxis and Desensitisation.—It is also possible to produce a condition of *anti-anaphylaxis*. If, for example, the small sensitising dose of horse serum is injected, and before anaphylaxis is established (*i.e.* some time before the tenth day) another injection consisting of a considerable quantity of horse serum is made, the animal is then found to be non-susceptible to further injections for a considerable period of time, although its serum during this period is capable of conferring passive

anaphylaxis. Eventually, however, the state of anaphylaxis becomes established. In a similar way non-susceptibility to a further dose of serum supervenes for a time when an animal recovers from anaphylactic shock. On the other hand, if anaphylaxis exists, the serious effects may be avoided by the injection of a small dose of serum, insufficient in itself to bring about typical symptoms, and then by the injection of graduated increasing doses. In this instance the refractory state is brought about by a process of *desensitisation* (*vide infra*) ; according to Morris, the factors concerned here may be largely non-specific, since saturation of antibodies is not chiefly responsible. Insensitiveness may also be induced by ether anæsthesia at the time the assaulting dose is given, and by drugs such as atropine or adrenaline, or by the injection of hypertonic saline, or of serum of a species other than that with which the animal has been sensitised, all given shortly beforehand.

Symptoms and Pathological Changes in Anaphylaxis.—With regard to the symptoms and pathological changes in anaphylaxis, two facts are of importance. The first is that in a given species of animal these are of the same nature, no matter by what antigen the condition has been produced. The second is that the anaphylactic phenomena taken as a whole vary according to the species of animal. This does not mean that the condition varies essentially in nature ; it probably indicates merely that different tissues are more susceptible in different animals. The symptoms of acute anaphylaxis in the guinea-pig have already been described, and the chief change found *post mortem* is an acute over-distension of the lungs which is due, as was first shown by Auer and Lewis, to a spasm of the muscle fibres in the fine bronchi and alveolar passages. In rabbits the acute symptoms are somewhat different, there being an absence of dyspnœa ; the chief post-mortem change is a great accumulation of blood in the venous system, and this results from contraction of the pulmonary arterioles. In the dog there are symptoms in connection with the alimentary canal, vomiting and evacuation of the bowels, followed by a shock-like fall of the blood pressure. *Post mortem*, the chief changes are found to be a great enlargement of the liver due to accumulation of blood and an intense engorgement of the portal area. In both the rabbit and the dog there is marked leucopenia, and this is attended by diminution in the coagulability of the blood. The presence of subserous capillary hæmorrhages is another feature. It may be noted that in both of these animals acute anaphylaxis is less readily brought about than in the guinea-pig, and usually requires for

its production more than one sensitising dose. A minor degree of sensitisation leads to a " protracted " or less acute anaphylaxis. Of acute fatal anaphylaxis in the human subject, comparatively few authentic cases have been recorded (Waldbott); but in one case carefully studied and described by Dean where death occurred in little more than one hour, the main symptoms and pathological changes corresponded with those observed in the dog.

Mechanism of Anaphylaxis.—While it is now admitted by all that anaphylaxis depends on an interaction of antigen and antibody (anaphylactogen and anaphylactin), there has been much diversity of opinion as to the site and mode of its occurrence. Two chief views have been put forward. According to one, the combination takes place in the tissues and there produces disturbance; according to the other, the poison is formed by the combination in the circulating blood and thus affects various parts of the body—the *cellular* and *humoral* theories respectively.

It has now been established that in an anaphylactic animal the antibody (anaphylactin) is present in the tissues, and toxic effects result when antigen is brought into relation with it. Schultz showed that isolated segments of the intestine of a sensitised animal responded by contraction in a specific way to the application of the antigen, and this result was confirmed, and amplified in many ways in extensive experiments by Weil and by Dale. Using the muscle of the uterus of the guinea-pig from which the blood had been removed by perfusion, Dale showed that when the specific antigen is brought into contact with it the muscle undergoes sudden contraction, which begins to pass off after a few minutes; thereafter the muscle is desensitised and does not respond further. He compared the effect to that of a powerful stimulant drug, and considered that it did not resemble enzyme action or any form of proteolysis etc. It has been shown by various workers that the anti substance in anaphylaxis corresponds closely in many respects with precipitin. In fact, some hold that the two substances are identical, since for the production of passive anaphylaxis it is not essential that the serum should be derived from an animal in the anaphylactic state, provided that it contains a precipitin for the test antigen. It is thus possible that, as Dale suggests, the phenomena may be due to a disturbance of the relations of the colloids of the muscle, short of precipitation. The view as to the reaction taking place in the tissues is in harmony with other results. The fact that in an actively sensitised guinea-pig

the symptoms appear at once on injection of the antigen, whereas in passive anaphylaxis a considerable time must elapse after the antiserum is injected before the animal develops maximal sensitiveness to the antigen, is a stumbling-block to the theory that a poison is formed by the interaction of the two substances in the blood. It seems, however, capable of explanation on the view that the interval of time is necessary for the antibody in the serum to be fixed in the tissues. The so-called "replacement" experiments, in which the blood of the sensitised animal is replaced by normal blood, lead to a similar conclusion as to the site of the reaction; in such experiments the animal is still anaphylactic on injection of antigen, and its separated tissues are so also. In addition, attempts to show that the blood of an animal in acute anaphylactic shock is toxic to another animal have given negative results. But blood obtained directly from the liver of dogs in a state of anaphylactic shock produces anaphylactic effects when introduced into the circulation of a normal dog. This is in accordance with the view that in these animals the liver is the chief seat of disturbance, since it has been found that in a sensitised dog in which the liver vessels are clamped shock does not occur on the injection of the foreign protein, but after releasing the clamps the usual effects develop (Manwaring).

The humoral theory that anaphylaxis is due to a toxin formed in the circulating blood by the two substances has been advanced in various forms. It was originally put forward by Richet, who called the toxic product "apotoxin." It is unnecessary to detail the various views, but reference may be made to the work of Friedberger, which included a very extensive analysis of the subject. He explained the phenomena as resulting from the process of digestion of protein introduced parenterally, the toxic agent being a disintegration product. He showed that the action of complement on a serum precipitate (antigen+precipitin) produced a toxic body which, on being separated from the precipitate and injected into an animal, produces the symptoms of anaphylaxis; this body he called *anaphylatoxin*. He showed also that anaphylatoxin is produced by the action of complement on bacteria treated with their antiserum, and also by the action of normal serum alone on bacteria, and even on coagulated serum. The possibility of proteolytic action by complement has, however, been called in question, and corresponding symptoms have been produced in other ways. For example, complement-containing serum on digestion with kaolin, agar, etc., has been found to acquire similar properties, and it is generally held now that the various anaphylatoxins are not specific in their action.

Dale and Kellaway have found that the effects produced by the anaphylatoxins differ from those in true anaphylactic shock,

evidence of injury to the vascular endothelium and lysis of platelets being more conspicuous in the action of all of them. Further, they showed that tolerance to anaphylatoxin can be acquired and that this does not involve desensitisation of the anaphylactic animal ; on the other hand, desensitisation of an anaphylactic animal does not make it non-susceptible to the action of anaphylatoxin. The phenomena of anaphylaxis, however, are very complicated, and while the combination of antigen with antibody in the tissues leads to the essential effects, the possibility that toxic bodies formed in the blood itself may play a part cannot be excluded ; this is more likely to be the case when death does not occur quickly. In anaphylaxis there is often a marked fall in complement, and this is apparently due to its fixation in the usual way by the combination of antigen *plus* antibody. There is evidence that the diminution in the coagulability of the blood, which is a noteworthy change in the rabbit and the dog, results from the setting free of anti-coagulants from the liver. The marked leucopenia, which is often a striking feature, is due to the accumulation of the leucocytes in the lung and other organs, in a similar manner to what is seen after the injection of a dose of peptone or other substances into the blood. Dale brought forward evidence which rendered it likely that the anaphylactic reactions are due, in part at least, to the effect of histamine liberated as a result of cell damage caused when the antigen reacts with the antibody in the tissues of the sensitised animal. This has been confirmed by Bartosch, Feldberg and Nagel in the following experiment. The anaphylactic reaction was produced in an isolated lung preparation of an actively sensitised guinea-pig by adding the antigen to artificial perfusion fluid passing into the pulmonary artery. The fluid issuing from the pulmonary vein of the preparation when in the state of anaphylactic shock was then perfused through a similar lung preparation of a normal guinea-pig and caused the latter also to pass into the state of acute distension characteristic of the anaphylactic reaction—the substance responsible for this effect being shown to be histamine derived from the lung tissue of the first animal.

Besredka considers that the sensitising and the toxic factors in the horse serum are not one and the same. He finds that serum heated to a certain temperature may still have the power of inducing the condition of anaphylaxis, but has lost the power of bringing about the toxic phenomena when injected into an anaphylactic animal. This result has, however, been explained by others as being due to the fact that the sensitising dose is so much smaller than the toxic dose (*vide supra*) on re-injection ; accordingly, the

effect of heat may be to reduce the latter below the fatal limit without having a corresponding effect on the sensitising dose.

Allergy.—It is still an open question to what extent the phenomena of anaphylaxis just described are of the same nature as the supersensitiveness or allergy to the products of the infecting organism, *e.g.* that to tuberculin, mallein, etc., manifested by patients suffering from disease; though in all probability they are at least similar in essence. It has been held as a distinction that this supersensitiveness in infections to bacterial products could not be transferred to another animal, and there is no conclusive evidence that this is possible in the case of tuberculin. According to one view, which appears to us to have most in support of it, the phenomena of supersensitiveness of tuberculous patients to tuberculin is due to the combination of the injected antigen with molecules of antibody resident in the tissue cells, the so-called " sessile receptors." In favour of this view is the observation of Rich and Lewis that tissue cultures *in vitro,* which have been derived from animals supersensitive to tuberculin retain this supersensitiveness after repeated subculturing. Friedberger, however, holds that the facts can be equally well explained by the combination, which occurs either locally or generally, of the antigen with antibody *in the serum*, which combination when acted upon by complement gives rise to the poisonous substance. Others, again, consider that there is not sufficient evidence that an antibody is concerned, and that local allergy is of a different nature. According to Dienes, specific supersensitiveness of the tissues is the first manifestation of reaction and is an indicator of immunisation. But Rich and others hold that in animals which are both immune and supersensitive, desensitisation may be effected without removing the immunity. At present it is not possible to make a definite statement on the subject. Further details are given in connection with the special chapters. There is no doubt that the supersensitive condition must play an important part in the clinical manifestations of many diseases. For example, the sensitiveness of tuberculous patients to tuberculin shows that the symptoms in this disease are evidently produced by the absorption from the tuberculous foci of a smaller amount of toxin than would be necessary to produce corresponding effects in a normal individual. And the sensitiveness of the conjunctiva in typhoid fever to the products of the bacillus suggests that in this disease also supersensitiveness plays an important part.

It appears at least likely that bacterial products set free in the body in an infection gradually produce a state of supersensitiveness to these products, which is closely similar to allergy if not to a true anaphylactic state. The phenomena of hay fever probably belong to the same class, being apparently the result of repeated absorption of vegetable proteins; a similar statement may apply to the sensitiveness to articles of diet. In all such cases, however, the possibility of a natural supersensitiveness as an *idiosyncrasy* must be considered. Supersensitiveness of infants to cow's milk has been recorded, and the history of cases indicates that such is due to the absorption from the alimentary tract of protein molecules, or, at least, of derivatives which may act as antigens. The possibility of such an occurrence is shown by the work of Ehrlich on the vegetable toxins, ricin and abrin (p. 218); and Walzer has demonstrated by sensitisation methods the presence in the serum of incompletely digested proteins after their ingestion by normal adults.

Atopy.—In a number of these conditions of idiosyncrasy in which supersensitiveness to certain substances appears clinically as hay fever or asthma, or susceptibility to certain articles of diet, it has been shown that the serum contains a "reagin," which when injected into the skin of a normal person may produce local sensitiveness, so that on introducing the substance later at the same site an inflammatory reaction develops (Prausnitz-Küstner reaction). Attempts to demonstrate neutralising properties in the reagin, or other characters of antibodies have failed, however. A certain amount of evidence has been brought forward that puerperal eclampsia is produced by the absorption of proteins from the placenta, which have the property of establishing an anaphylactic state. It must be admitted, however, that our knowledge as to such problems is still very defective.

Recently a striking phenomenon has been described by Shwartzman, which is related to allergy. The intradermal injection of bacteria or their products in rabbits causes local changes such that if after an interval, *e.g.* of twenty-four hours, an intravenous injection of filtrates from cultures of the same or other organisms be given, an acute reaction is provoked in the treated area of skin; this is characterised by hæmorrhage and necrosis. The effect may also be provoked by starch given intravenously (Freund) or when the animal has previously been rendered supersensitive to some antigen an intravenous injection of the latter may provoke the reaction at the prepared site. Accordingly, a mechanism is suggested which may account for

the development of focal lesions owing to some intercurrent infection, although the explanation is obscure.

The Serum Disease in Man.—Apart from acute anaphylaxis in man referred to above, symptoms of milder degree are not infrequently observed after the injection of foreign serum for therapeutic purposes; they were first fully described as a syndrome by Pirquet and Schick. There is here also a period of incubation, of six to twelve days on the average, but it may be three weeks; after which, in a proportion of cases, a group of characteristic symptoms appears. The proportion affected depends on the dose of serum; when amounts of 100 to 1000 c.c. are given nearly all cases develop serum disease; after a dose of under 10 c.c. about 10 per cent. are affected. There may be as prodromal symptoms, swelling and tenderness at the site of injection, and in the corresponding lymphatic glands, and the spleen may be enlarged. Thereafter general exanthemata appear; these are usually of an urticarial type, but may be erythematous or morbilliform. There is usually moderate pyrexia of a remittent type, and sometimes œdema and slight albuminuria are present; occasionally there are pains in the joints; there is also in children often leucopenia, due to a fall in the number of polymorphonuclear leucocytes. These symptoms last for a few days and then disappear. Sometimes after an interval, which may extend to two weeks, there is a relapse. Such are the phenomena of the serum disease after a single injection of the foreign serum. There are, however, two other types of reaction described by Pirquet and Schick, namely, the *immediate* and the *accelerated* reactions. The immediate reaction is seen when a large dose of serum has been administered, and then after a certain interval of time another dose of serum is injected. This interval is usually from twelve days to eight weeks, though sometimes as long as six months. The symptoms of the immediate reaction, which appear shortly after the injection, or at least within twenty-four hours, are an intense œdema locally, general exanthemata and pyrexia, though the general phenomena are often little marked. The symptoms pass off comparatively quickly, usually within twenty-four hours. When the injection has been given intravenously, the chief symptoms are dyspnœa, with pallor or cyanosis, fall in the blood pressure, with feeble pulse, asthmatic symptoms, with cough and sometimes vomiting and severe headache—all setting in within a few minutes to two hours. The accelerated reaction is also seen after a second injection, and it may occur from six weeks up to many months after the first injection. In the case of the accelerated reaction there is

an incubation period, but it is shorter than in the case of the first injection, being usually from five to seven days; the symptoms resemble those in the ordinary reaction as described above, but are of rather more acute onset and last a shorter time. In the interval from about the sixth week to the sixth month, there may occur both the immediate reaction, and also a few days later an accelerated reaction. The occurrence of serum disease following the injection of fresh human serum from other individuals has been described (Dooley).

The phenomena of the serum disease in all probability depend upon the development of a reaction-body or antibody, as above described, though this view has been called in question by some writers. Cases are recorded where symptoms have occurred almost at once following the first injection, or after a time too short to correspond to a true incubation period. These may, however, be examples of "natural" supersensitiveness; they have occurred especially in persons with a history of supersensitiveness to contact with horses. But the facts described above, especially with regard to the phenomena after a second injection, point strongly to an antibody being concerned in the reaction. In recent times the intracutaneous injection of a small quantity of the serum has been used as a test for supersensitiveness (*vide infra*), and this apparently depends on an antigen *plus* antibody reaction.

Very serious local effects of the nature of the Arthus phenomenon (p. 261) have also been recorded rarely as the results of repeated injections of foreign serum into the tissues (Tumpeer, Ross).

Practical Results—Desensitisation.—In view of the common use of curative serum, anaphylaxis has come to have considerable practical importance, especially in connection with intravenous injection, as by this route the dangerous dose is a fraction of that by subcutaneous injection. With regard to the possibility of there being a primary or *natural* supersensitiveness, inquiry should be made as to tendency to asthma or hay fever, or sensitiveness to the presence of horses in the vicinity, as these have been found to be associated conditions, and the existence of Graves' disease has been recorded as another. Then with regard to the *acquired* variety, information should be obtained as far as possible regarding previous serum injections. The existence of supersensitiveness can, however, be demonstrated by the test for skin allergy. A small quantity, say 0·05 c.c., of a 1 : 10 dilution of sterile horse serum with saline is injected by a hypodermic syringe into the dermis—not subcutaneously—a similar

quantity of saline alone being injected into another site by way of control. The minute local swelling which results from the presence of the fluid soon passes off. But in the case of a positive reaction there occurs, usually within five to thirty minutes, an urticarial patch, which may be followed by a distinct vesicle and is often surrounded by an erythematous area, an inch or more in diameter. Another method consists in allowing one drop of 1:10 dilution of the serum to fall into the conjunctival sac ; a positive result is indicated by hyperæmia, swelling, itching or pain (which should be treated by instillation of adrenaline). If no reaction occurs within forty minutes in these tests the absence of supersensitiveness may be inferred. But a negative result, at least in the skin test, does not exclude the possibility of serious symptoms when the serum is administered. If a positive reaction is obtained, the attempt should be made to desensitise the patient, *i.e.* to produce anti-anaphylaxis ; and this is accomplished by introducing initial small doses of serum and then gradually increasing them. Even then, care should be used in injecting the serum intravenously or intraspinally, as desensitisation in the human subject is uncertain.

The initial desensitising dose is 0·01 c.c. given subcutaneously, and this amount is doubled every half-hour. If no reaction follows the administration of 1 c.c., the subsequent doses are given intravenously, commencing with 0·1 c.c. and doubling the dose every half-hour till the total dose has been given. Where it is desired to inject the serum intraspinally the above procedure should be followed till an intravenous dose of 10 c.c. has been given ; then the same amount may be injected intraspinally. Such a method, however, takes a considerable number of hours and is not justifiable in a case of tetanus, where a large amount of serum should be given intravenously or intrathecally as soon as possible. The following method, given in a War Office memorandum, should be followed : 5 c.c. of the antiserum are diluted with 50 c.c. of normal salt solution. Of the mixture 1 c.c. is injected intravenously ; this is followed four minutes later by 3 c.c., two minutes later by 10 c.c., and two minutes later again by 25 c.c. Then after ten to fifteen minutes the full dose may be given intravenously or intrathecally. The doses mentioned are most suitably given by the gravitation method of injecting serum intravenously or intraspinally, and the serum should be diluted with saline.

If any anaphylactic symptoms appear (p. 270), the administration must be temporarily stopped and then cautiously resumed. Adrenaline and atropine are the most efficient drugs. In all cases the administration of serum by the methods mentioned should be carried out slowly and with caution. Anaphylaxis is sometimes a real danger, but the risks, when we take

into account the necessity for the prompt treatment of tetanus, have been exaggerated. We may add that the repeated subcutaneous injections for preventive purposes of the usual quantity of 3 c.c. of serum are unattended by any danger. It may also be stated that in relation to anaphylaxis it is only the *amount of serum* and the *species* from which it is drawn which matter—the antitoxic value is not a factor. Accordingly it has been suggested that when antiserum is given solely with a view to prophylaxis, *e.g.* of diphtheria, it should be derived from another species such as the goat or ox; thus the development of supersensitiveness to horse serum is avoided, in case later on a potent dose of antibody contained in the latter should be required for treatment.

CHAPTER VII

PYOGENIC COCCI AND OTHER BACTERIA ASSOCIATED WITH INFLAMMATORY AND SUPPURATIVE CONDITIONS

This subject is an exceedingly wide one, and the organisms to be considered produce a variety of pathological conditions which in their general characters and results are widely different. Thus, in addition to the commoner suppurative processes, various inflammatory conditions, endocarditis, septicæmia and pyæmia, require consideration. The following general statements may be made in introducing the subject. In the first place, various organisms may produce substantially the same type of lesion, and not infrequently more than one organism may be present. In the second place, the same organism may produce widely varying results under different circumstances—at one time a local inflammation or abscess, at another multiple abscesses or a general septicæmia. The principles on which this diversity in results depends have already been explained (p. 172). Furthermore, there are conditions like acute pneumonia, epidemic meningitis, scarlatina, etc., which have practically the characters of specific diseases, and yet, as regards their essential pathology, belong to the same class.

It may be well to emphasise some of the chief points in the pathology of these conditions. In *suppuration* the two main phenomena are—(*a*) a progressive emigration of leucocytes, chiefly of the polymorphonuclear (neutrophile) variety, and (*b*) a liquefaction or digestion of the supporting elements of the tissue along with necrosis of the cells of the part. The result is that the tissue affected becomes replaced by pus. A suppurative inflammation is thus to be distinguished on the one hand from an inflammation without destruction of tissue, and on the other from necrosis or death *en masse*, where the tissue is not liquefied, and leucocyte accumulation may be slight. When, however, suppuration is taking place in a very dense fibrous tissue, liquefaction may be incomplete, and a portion of dead

tissue or slough may remain in the centre, as is the case in boils. When suppuration occurs in a serous cavity the two chief factors are the progressive leucocytic accumulation and the disappearance of any fibrin which may be present.

Many experiments have been performed to determine whether suppuration can be produced in the absence of micro-organisms by various chemical substances, such as croton oil, nitrate of silver, turpentine, etc.—care, of course, being taken to ensure the absence of bacteria. The general result has been that in certain animals and with certain substances suppuration may occur, the pus being free from bacteria. Buchner showed that suppuration may be produced by the injection of dead bacteria, *e.g.* sterilised cultures of *Bacillus pyocyaneus*, etc., and, according to Vaughan, bacterial protein *per se* may produce suppuration and necrosis of tissue. The subject has now more a scientific than a practical interest, and the general statement may be made that practically all cases of true suppuration met with clinically are due to the action of living micro-organisms. It may be noted here that the injection of certain substances into a serous cavity, *e.g.* aleuronat, peptone, sodium nucleate, etc., may produce within twenty-four hours an abundant exudate containing large numbers of polymorph leucocytes, and in this way such cells can be obtained unmixed with other blood cells for experimental studies of their immunological and other properties.

The term *septicæmia* is applied to conditions in which the organisms multiply within the blood and give rise to symptoms of general poisoning, without, however, producing abscesses in the organs. The organisms are usually more numerous in the capillaries of internal organs than in the peripheral circulation, but the application of methods of blood culture has shown that they can be detected in the peripheral blood. It should be remembered that bacteria may also be demonstrable in the blood as a result of their "overflow" from an infective lesion in the tissues, and such blood infection—*bacteriæmia*—does not necessarily connote a true septicæmia or multiplication of organisms within the vascular system. The essential fact in *pyæmia*, in contrast with septicæmia, is the occurrence of multiple abscesses in internal organs and other parts of the body. In most of the cases of typical pyæmia, common in pre-antiseptic days, the starting-point of the disease was a septic wound with bacterial invasion of a vein, leading to thrombosis and secondary embolism. Multiple foci of suppuration may be produced, however, in other ways, as will be described below.

A considerable number of species of bacteria have been found in acute inflammatory and suppurative conditions, and most of these have been proved to be causally related. But it should be recognised that many organisms when experimentally introduced into the tissues are capable of producing an acute inflammation and even pus formation; only certain species, however, are characteristically associated with such lesions under natural conditions. These organisms now to be described are usually known as *pyogenic*.

Ogston, who was one of the first to study this question (in 1881), found that the organisms most frequently present were micrococci, of which some were arranged irregularly in clusters (staphylococci), whilst others formed chains (streptococci). He noted that the former were more common in circumscribed acute abscesses, the latter in spreading suppurative conditions. Rosenbach shortly afterwards (1884), by means of cultures, differentiated several varieties of micrococci, to which he gave special names: e.g. *Staphylococcus pyogenes aureus*, *Staphylococcus pyogenes albus*, *Streptococcus pyogenes*. Suppuration may be produced, however, by various other species, *e.g.* different types of staphylococci and streptococci (*vide infra*), *Micrococcus tetragenus*, pneumococcus, meningococcus, gonococcus, *Bacillus coli*, pneumobacillus (Friedländer), *Bacillus pyocyaneus*, *Bacillus proteus*, *Actinomyces*, *Bacillus mallei*, etc. Even the typhoid and paratyphoid bacilli, which primarily are not pyogenic organisms, may be associated with suppurative lesions occurring as complications or sequelæ of enteric fever. *Bacillus influenzæ* may also occur in secondary inflammatory and suppurative conditions. Various anaerobic bacteria are concerned in the production of an inflammation which is often associated with œdema, hæmorrhage, or necrosis (*vide* Chapter XX).

Mode of Entrance and Spread—Primary Infection.—Many of the pyogenic organisms have a wide distribution in nature, and many also are present on the skin and mucous membranes of healthy individuals. Staphylococci and streptococci are constant commensals in the mouth, nose, and throat. Even the typical pyogenic streptococci may occur normally in the throat, and particularly in the tonsillar crypts. The entrance of these organisms into the deeper tissues when a surface lesion occurs can be readily understood. Their action will, of course, be favoured by any condition of depressed vitality. Though in normal conditions the blood is bacterium-free, we must suppose that from time to time a certain number of such organisms gain entrance to it from trifling lesions of the skin or mucous

surfaces, the possibilities of entrance from the latter being especially numerous. In most cases they are killed by the action of the plasma or phagocytes, and no lesion results. If, however, there be a local weakness, they may settle in that part and produce suppuration, and from this other parts of the body may be infected. Such a supposition as this is necessary to explain many inflammatory and suppurative conditions met with clinically. In some cases of multiple suppurations due to staphylococcus infection, only an apparently unimportant surface lesion is present; whilst in others no lesion can be found to explain the origin of the infection. The term *crypto-genetic* has been applied by some writers to such cases in which the original point of infection cannot be found, but its use is scarcely necessary.

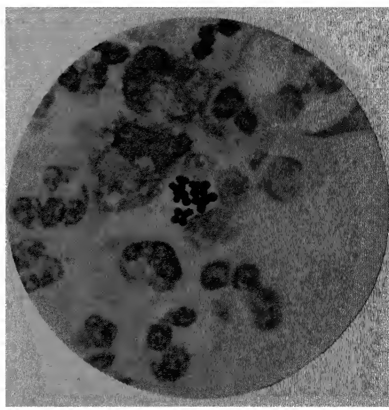


FIG. 26.—Staphylococci in Pus. Stained by Gram's method. ×1000.

The *paths of secondary infection* may be conveniently summarised thus: *First*, by lymphatics; in this way the lymphatic glands may be infected, and also serous sacs in relation to the organs where the primary lesion exists. *Second*, by natural channels, such as the ureters, the spread being generally associated with an inflammatory condition of the lining membrane. In this way the kidneys may be infected. *Third*, by the blood vessels: (*a*) by a few organisms gaining entrance to the blood from a local lesion, and settling in a favourable nidus or damaged tissue, the original path of infection often being obscure; (*b*) by a septic phlebitis with suppurative softening of the thrombus and resulting embolism; and (*c*), by a direct extension along a vein producing a spreading thrombosis and suppuration within the vein. In this way suppuration may spread along the portal vein to the liver from a lesion in the alimentary canal, the condition being known as pylephlebitis suppurativa.

The Staphylococci

Staphylococcus pyogenes aureus (*Staphylococcus aureus*, Rosenbach).—*Microscopical Characters.*—This organism is a spherical coccus 0·8 to 0·9 μ in diameter, which tends to grow in irregular clusters or masses (Figs. 26, 27), since after a coccus has divided the subsequent planes of division of the daughter cells bear no fixed relation to each other; the opposed surfaces

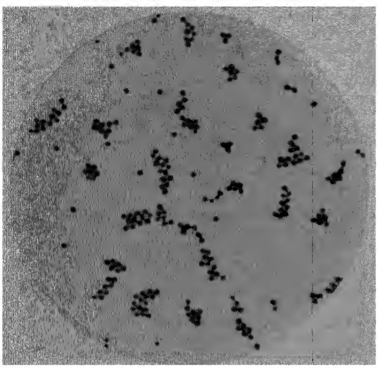


Fig. 27.—*Staphylococcus aureus*, young culture on agar, showing clumps of cocci. Stained by Gram's method. ×1000.

Fig. 28.—Stab culture of *Staphylococcus aureus* in gelatin—17 days old; showing liquefaction of the medium and characters of growth. Natural size.

of adjacent cocci may be flattened. Single organisms, pairs or short chains may also be observed. The organism is non-capsulated, non-motile, and does not form spores. It stains readily with all the basic aniline dyes, and retains the colour in Gram's method (Plate I., Fig. 1).

Cultivation.—Growth occurs under aerobic conditions, but the organism is also a facultative anaerobe. It grows readily in all the ordinary media at room temperature, though much more readily at the temperature of the body. The temperature range for growth is 10° to 42° C., the optimum temperature 34° to 36° C. On *agar*, a stroke culture forms a line of abundant yellowish growth, with smooth shining surface, well developed after twenty-four hours at 37° C. Later it becomes bright orange in colour, and resembles a streak of oil paint. Single colonies on

the surface of agar are circular discs of similar appearance, which may reach 3 to 4 mm. after twenty-four to forty-eight hours. In a primary culture from pus the colonies after twenty-four hours at 37° C. may be much smaller and pigment mav be absent, so that to the naked eye they resemble a growth of streptococci (*vide infra*). (But if the growth is caused to heap up into a mass by drawing a platinum loop along the surface of an agar slope culture, then the pigmentation often becomes apparent.) Pigment production is manifest when the organism is growing in the presence of oxygen, and is most pronounced in cultures kept at room temperature and exposed to light. The pigment is a lipochrome and is stated to be allied to carotene. On *blood agar* a clear zone of hæmolysis is noted round the colonies, due to the diffusible hæmolysin produced by the organism. In stab cultures in *gelatin* a streak of growth is visible on the day after inoculation, and on the second or third day liquefaction commences at the top. As liquefaction proceeds, the growth falls to the bottom as a flocculent deposit, which soon assumes an orange-yellow colour, while a yellowish film may form on the surface, the fluid portion still remaining turbid. Ultimately liquefaction extends out to the wall of the tube (Fig. 28). In *gelatin plates* colonies may be seen with the low power of the microscope in twenty-four hours, as little spheres, somewhat granular on the surface and of brownish colour. On the second day they are visible to the naked eye as whitish-yellow points, which in typical strains afterwards become more distinctly yellow. Liquefaction occurs around these, and small cups are formed, at the bottom of which the colonies form yellowish masses. On *coagulated serum*, pigment production is usually marked and liquefaction or softening of the medium occurs. On *potato* the organism grows well at ordinary temperature, forming a somewhat abundant layer of orange colour. In *broth* it produces usually a uniform turbidity, which afterwards settles to the bottom as an abundant deposit and assumes a brownish-yellow tint. It grows readily in *milk* acidifying the medium and producing coagulation. The fermentative properties are referred to later. *Staphylococcus aureus* has considerable tenacity of life outside the body, and withstands drying even for several weeks. The thermal death-point is approximately 62° C., but some strains resist higher temperatures, even 70° C. for half an hour. Laboratory cultures remain viable for months.

The *Staphylococcus pyogenes albus* (*Staphylococcus albus*, Rosenbach) is similar in general characters, with the exception

that its growth on all the media is white. A similar organism, called by Welch *Staphylococcus epidermidis albus*, is practically always present on the skin. It may be said that in general the *albus* type of staphylococcus is weaker in its biochemical, toxic, and pathogenic properties than *Staphylococcus aureus*, and strains normally present in the skin liquefy gelatin slowly and are non-hæmolytic.

The *Staphylococcus citreus*, which is less frequently met with, differs in the colour of the cultures, being lemon-yellow, and is usually less virulent than the other two.

Other varieties of staphylococci have also been described, *Staph. cereus flavus* and *Staph. cereus albus*, so designated in virtue of their wax-like growth, and the coloration produced. They do not liquefy gelatin. These types are of rare occurrence.

Staphylococcus ascoformans is a type of staphylococcus similar to *S. aureus* and associated with equine "botryomycosis." In the lesions it forms large clusters, and the cocci are capsulate. Similar appearances have been met with rarely in human lesions (Berger).

Biochemical Reactions of the Staphylococci. — These organisms ferment various carbohydrates, *e.g.* glucose, lactose, maltose, mannitol, with acid formation, and attempts have been made to classify them by such reactions, though without satisfactory results. It is noteworthy, however, that the pyogenic strains usually ferment mannitol, whereas the *albus* types found on the skin do not give this reaction.

Classification of the Staphylococci.—As indicated above, these organisms have generally been classified according to their growth-characters. Fermentative reactions have not yielded clear-cut results for the identification of different types. Some workers have observed variation as regards pigmentation, consistence, and "smoothness" or "roughness" of colonies (p. 39). Thus Bigger and his co-workers have described the appearance of a white variant in old cultures of the *aureus* type and various observers have found that the *albus* type may be derived from the *aureus*. Hine has classified the staphylococci into two main groups, according to their reactions in agglutinin-absorption tests with specific antisera: (1) *S. pyogenes*, which includes both *aureus* and *albus* types, and (2) *S. epidermidis*, which is mainly of the *albus* type. This work would indicate that the colour of growths is of less importance in biological classification than has hitherto been assumed. It should be noted, however, that certain other characters are more pronounced among the *aureus* than among the *albus* types—*e.g.* liquefaction of gelatin, sugar fermentation, hæmolysin production, and pathogenicity—and it seems justifiable to adhere to the original nomenclature and classification.

Staphylococci occur as commensal organisms on the skin, in the mouth, throat, auditory meatus, nose, and conjunctiva, the prevalent type being *Staphylococcus albus*. They may also be present in the anterior urethra, stomach, and occasionally in

the intestine. These organisms are therefore found in the air, in dust, on clothing, etc. Staphylococci have also been found in an apparently latent state in the tonsils and in lymphatic glands.

Toxins of the staphylococci.—As indicated above, these organisms, particularly the *aureus* type, produce a diffusible hæmolysin which can be demonstrated in filtrates of suitable broth cultures or by suspending a twenty-four hours' agar culture in a small amount of saline and then removing the organisms from the fluid by centrifuging at high speed (Bigger, Boland, and O'Meara). This product is usually thermolabile at 55° C. It corresponds to the bacterial exotoxins, and a neutralising antitoxin can be obtained by immunising animals with it. Culture-filtrates may also possess the power of killing leucocytes and the active principle (as in the case of other bacteria yielding a similar product) has been designated " leucocidin." The leucocidin can be demonstrated conveniently by the methylene blue reduction test: rabbit leucocytes are obtained by intrapleural injection of aleuronat (*vide* p. 275), washed free from serum and suspended in normal saline; these cells normally reduce methylene blue under partially anaerobic conditions but after they have been acted on by a leucocidal toxin lose this property.

Recently the toxic effects of the staphylococci have been carefully studied and it has been shown that culture-filtrates possess a necrotising effect when injected in small quantity into the skin of animals, and when injected intravenously rapidly produce a lethal result, acting mainly on the heart and capillary vessels of the lung. The necrotising substance has been called " necrotoxin." Thus four apparently different toxic effects have been demonstrated due to diffusible products analogous to the exotoxins generally, and immunisation of animals with filtrates yields an immune serum which annuls these various toxic properties. The question has arisen whether these effects are due to a single toxin or to different toxic substances. The evidence available indicates their diversity. According to Burky, the lethal factor is stable as compared with the lability of the hæmolysin. Strains vary quantitatively in their toxicity and there is no strict quantitative correlation as regards the production of the different factors. There is also no definite correlation between their pathogenicity or virulence and degree of toxicity.

Glenny and Stevens have shown that some strains of *Staphylococci* may produce two different hæmolysins (designated α and β)

for which separate antitoxins can be obtained. The α hæmolysin is indicated by its rapid activity at 37° C. towards rabbit and sheep erythrocytes. The β hæmolysin is characterised by its continuous and progressive action on sheep's red cells at room temperature after preliminary incubation at 37° C It is of special interest that strains of staphylococci from animal lesions, *e.g.* bovine mastitis, yield the β hæmolysin (Minett).

Destructive action by staphylococcal filtrates towards human leucocytes as observed microscopically (Panton and Valentine) is apparently due to a leucocidal factor different from that which annuls the power of rabbit leucocytes to reduce methylene blue (*vide supra*) and J. Wright has found that the former is identical in thermolability (at 40° C.) with the α hæmolysin and differs from the β hæmolysin which is stable at 56° C According to Valentine, however, the leucocidin demonstrable with human leucocytes is different from the α hæmolysin

A cutaneous reaction in the human subject analogous to the Dick and Schick reactions (*vide* pp. 302, 391) may be elicited by intradermal injection of staphylococcal filtrate, but the significance of positive and negative results in this test has not yet been fully assessed. Anti-hæmolysin has been demonstrated in normal human blood (Bryce and Burnet), and this tends to increase in infected subjects

A further toxic effect of the staphylococci has been established in recent years: cases of food-poisoning have been recorded which have been due to toxin produced by these organisms growing in the particular article of food (Jordan). Milk from cows affected with staphylococcal mastitis has been responsible in one outbreak (Crabtree and Litterer). The illness has usually been an acute gastro-enteritis developing after an incubation period of a few hours. Jordan and Hall reproduced the condition in human volunteers by administering *per os* small quantities of culture-filtrates of staphylococci isolated from incriminated food stuffs. Woolpert and Dack obtained similar results in *rhesus* monkeys. Apparently laboratory animals are not readily susceptible to this toxin when introduced into the stomach but symptoms of poisoning (*e.g.* gastro-enteritis) can be produced when the acidity of the stomach contents is neutralised at the time of administration of the filtrate (Borthwick). The toxin is inactivated by even a slight degree of acid or alkali, but may resist boiling for some time, not being completely inactivated after thirty minutes. This toxic factor is apparently distinct from the other toxins present in filtrates, and is formed by only a minority of strains (Dolman).

The occurrence of endotoxic substances has also been referred to by various workers but they are apparently relatively weak in action and formed in small quantity.

Certain strains of staphylococci mainly of the *aureus* type

produce an enzymic substance which coagulates citrated rabbit plasma, acting in a manner similar to thrombin (R. Cruickshank). This can be demonstrated by adding a small quantity of young culture to citrated plasma and incubating at 37° C. for three hours. This "coagulase" is highly thermostable. While its production is a feature of the pathogenic strains its actual significance is doubtful. Menkin and Walston have endeavoured to ascertain whether the fibrinous thromboses observed in lymphatic vessels following the intradermal injection in rabbits of filtered cultures is due to coagulase. Their findings indicate, however, that the rapid obstruction of lymphatic channels is produced by the necrotoxin and not by coagulase.

Walbum's medium is suitable for the production of the staphylococcal toxins: it consists of an ox heart extract in which are incorporated 0·5 per cent. Witte's peptone, 0·2 per cent. potassium dihydrogen phosphate and 0·03 per cent magnesium sulphate (adjusted to a pH of 6·8) The growth is obtained in an atmosphere of 20 per cent. carbon dioxide. The following method for preparing staphylococcal toxin has been used by Parish and Clark: 2 per cent nutrient agar is diluted with Parker's buffered broth (equal parts of M/15 potassium dihydrogen phosphate solution—pH 7·4—and ordinary sugar-free meat infusion broth containing 4 per cent. Witte's peptone) until the concentration of agar is 0·8 per cent The medium is then sloped in suitable containers. The culture is grown in an atmosphere of 25 per cent. carbon dioxide. After forty-eight hours' growth more buffered broth is added to the culture, and growth is continued for twenty-four to seventy-two hours, as before in an atmosphere of 25 per cent. carbon dioxide.

Staphylococcal toxin, like other exotoxins, can be changed to *toxoid* by treatment with formaldehyde and in such inactivated form can conveniently be used for immunisation. Parish and his co-workers have found that rabbits immunised with staphylococcal toxoid exhibit a high degree of resistance to living cultures of the organism when administered subcutaneously or intravenously. The immunity produced is, of course, essentially antitoxic. This toxoid has been used with some success in the treatment of localised staphylococcal lesions, *e.g.* furunculosis, sycosis. According to Dolman clinical improvement is accompanied by a rise in the antitoxic titre of the patient's serum. Murray has estimated the average antitoxic value of human blood and compared it with that following immunisation with toxoid: a considerable increase was observed —from less than 1 unit to 7·5 or more units per c.c. of blood. For therapeutic purposes a series of about six graded injections of toxoid (0·05 c.c.–0·5 c.c.) into the deltoid muscle has been recommended. Smith's results in a controlled series of cases of

furunculosis, however, indicate that the content in antibody of the patient's blood is a relatively unimportant factor in this condition.

Experimental Inoculation.—It may be stated at the outset that the occurrence of suppuration depends upon the number of organisms introduced into the tissues, the dose necessary varying not only in different animals, but also in different parts of the same animal—a smaller number producing suppuration in the anterior chamber of the eye, for example, than in the peritoneum. The virulence of the organism also may vary, with correspondingly variable results on experimental inoculation.

Staphylococcus aureus, when injected subcutaneously in suitable numbers, produces an acute local inflammation, which is usually followed by suppuration. If a large dose is injected, the cocci may enter the blood stream in sufficient numbers to cause secondary suppurative foci in internal organs. Intravenous injection, in rabbits for example, produces results which vary according to the quantity used. If a relatively small quantity be injected, the cocci gradually disappear from the circulating blood; some become destroyed, while others settle in the capillary walls in various parts and produce minute abscesses. These are most common in the kidneys, where they occur both in the cortex and medulla as minute yellowish areas surrounded by zones of intense congestion and hæmorrhage. Similar small abscesses may be produced in the heart wall, lungs, liver, under the periosteum and in the interior of bones, and occasionally in the striped muscles. Very rarely indeed, on experimental injection, do the cocci settle on the healthy valves of the heart. If, however, when the organisms are injected into the blood, there be any traumatism of a valve, or of any other part of the body, they show a special tendency to settle at these weakened points.

Experiments on the *human subject* have also proved the pyogenic properties of these organisms. Garré inoculated scratches near the root of his finger-nail with a pure culture, a small cutaneous pustule resulting; and by rubbing a culture over the skin of the forearm he caused a carbuncular condition which healed only after some weeks. Confirmatory experiments of this nature were made by Bockhart, Bumm, and others.

When tested experimentally, the *Staphylococcus albus* has practically the same pathogenic effects as *Staphylococcus aureus*, though usually of less virulence; it rarely gives rise to severe infection. The common commensal strains of the *albus*

type may be practically non-pathogenic on experimental inoculation.

The effects produced by the experimental inoculation of the staphylococcal toxins have been dealt with above.

Lesions in the Human Subject.—The *Staphylococci* are the most common causal agents in localised abscesses, in pustules of the skin, blepharitis, carbuncles, boils, etc., acute suppurative periostitis and osteomyelitis; they also occur frequently in suppurating wounds, catarrhs of mucous surfaces, ulcerative endocarditis, urinary sepsis, and in pyæmic conditions. They are occasionally the causative organism of septicæmia. Reference has been made above to food poisoning by staphylococcal toxin, and certain of the staphylococcal lesions are also dealt with later.

Immunity.—Animals can be immunised experimentally by repeated and graded doses of killed or living organisms, and various specific antibody reactions can be demonstrated with immune sera, *e.g.* opsonic action, agglutination, but such sera do not exhibit *immune* bactericidal effects. Reference has been made above to the grouping of staphylococci on a basis of immunity reactions and antigenic structure but this classification cannot be directly correlated with other characters. While the saprophytic staphylococci are serologically heterogeneous the pathogenic forms show more homogeneity in this respect. As in various other bacterial groups, complex specific carbohydrates enter into the antigenic composition of the staphylococci (Julianelle and Wieghard; Hoffstadt and Clark), and these substances when isolated from the bacterial substance yield specific reactions with antisera. The relationship of specific carbohydrates to other characters of the organisms requires further study.

Staphylococcal vaccines have been extensively used for the prophylaxis and treatment of recurrent and chronic infections, *e.g.* furunculosis, but the results recorded by various observers are somewhat contradictory. Recently toxoid preparations have been advocated for therapeutic purposes in similar conditions (*vide supra*). Immune sera with antibacterial properties have been applied in the treatment of various staphylococcal infections but without satisfactory results. As already indicated an antitoxic serum can be obtained which neutralises the exotoxins of these organisms; on rational grounds such serum might be applicable in the treatment of the acute and toxic forms of staphylococcal infection, but its therapeutic value has not yet been fully established.

The Streptococci

The usual type of streptococcus found in suppurative conditions is *Streptococcus hæmolyticus* and this will be considered first.

Streptococcus hæmolyticus (classical variety—*Streptococcus pyogenes*, Rosenbach).—This organism (Plate I., Fig. 1) is a coccus, usually spherical, 0·7 to 1 μ in diameter, and occurring in chains composed generally of ten or more individuals (Figs. 29, 30). The chains vary somewhat in length, depending largely on the environment. As division may take place in many of the cocci at the same time, the appearance of a chain of diplococci is often met with. When microscopic preparations are made from cultures on solid media, chain formation may not be observed; in fluid media, however, chains are usually well developed (hence the term "*longus*" was at one time applied to this variety). In young cultures the cocci are fairly uniform in size, but after a time they present considerable variations, many swelling up to twice their normal diameter. These are to be regarded as involution forms. The organism is non-motile and non-sporing. The cocci are not capsulate as a rule, though a capsulate variety, "*S. epidemicus*" (reported in milk-borne septic sore throat) has been described. They stain readily with basic dyes and are Gram-positive. This reaction, however, is not so marked as in the staphylococci, and individual cocci may show only partial Gram-positive staining or even react negatively.

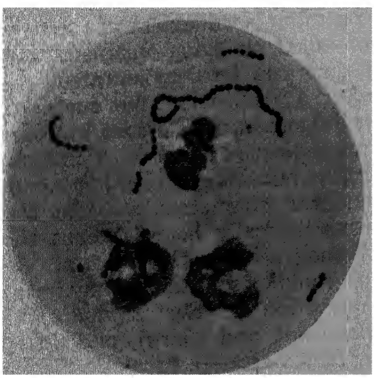


FIG. 29.—Streptococci in acute suppuration. Stained by Gram's method. ×1000.

Cultivation.—In cultures outside the body *Streptococcus hæmolyticus* grows less abundantly than the staphylococci, and also dies out more readily, being in every respect a more delicate organism. It grows aerobically or as a facultative anaerobe on the ordinary culture media, *e.g.* nutrient agar, the optimum temperature being about 37° C. Most strains do

not grow at room temperature. The addition of blood or serum to the medium enhances growth, and blood-agar is a convenient medium for routine cultivation (*vide infra*).

On *agar*, growth takes place along the stroke as a collection of small greyish-white circular discs of semi-opaque appearance, which tend to remain separate (Fig. 31). The separate colonies remain small, not exceeding 1 mm. in diameter after twenty-four hours' growth. Under a low power of the microscope they have a granular appearance. Cultures on agar kept at the

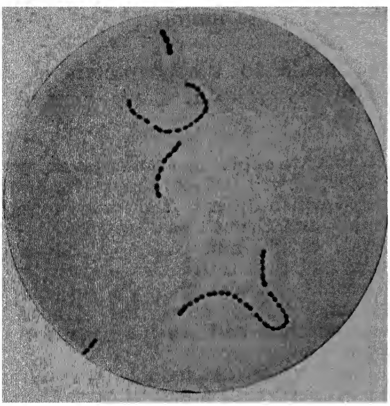


FIG. 30.—*Streptococcus haemolyticus* young culture showing chains of cocci. Stained by Gram's method. × 1000.

FIG. 31.—Culture of *Streptococcus haemolyticus* on an agar plate, showing numerous colonies—three successive strokes. Twenty-four hours' growth. Natural size.

body temperature may often be dead after ten days. On *blood agar*, the colonies are somewhat larger and are surrounded by a clear zone of laking or hæmolysis due to the hæmolysin produced by the organism. This presents a striking cultural character—hence the specific name *hæmolyticus*. Hæmolysis occurs most readily under partially anaerobic conditions. In nutrient *gelatin* (if growth occurs at room temperature) a stab culture shows about the second day a thin line, which in its subsequent growth is formed of a row of minute rounded colonies of whitish colour; these may be separate at the lower part of the stab. They do not usually exceed the size of a small pin's head, this size being reached about the fifth or sixth day. The growth

does not spread on the surface, and no liquefaction of the medium occurs. In *milk* a strongly acid reaction is produced, but no clotting of the medium. This organism may ferment with acid-production glucose, lactose, saccharose, salicin, and trehalose ; some strains also ferment mannitol ; it produces no fermentation of sorbitol, inulin, or raffinose ; but different strains vary as regards their fermentative reactions. In *broth*, growth forms numerous minute granules which afterwards fall to the bottom, the deposit, which is usually not very abundant, having a sandy appearance. The appearance in broth, however, presents variations, and if strains are repeatedly subcultured in this medium the growth becomes more uniformly turbid and less granular.

Further reference will be made to the characters of *S. hæmolyticus* in connection with the group of streptococci as a whole.

The *colony appearances* of strains are subject to a certain amount of variation, more manifest after growth has continued for some days. Variants can be separated which differ in the " roughness " or " smoothness " of the colonies, and Griffith has described three colony types : (1) opaque, flat, rough, and coherent, (2) non-coherent with an opaque centre and thin translucent border, and (3) large translucent and mucoid. Though there is no exact correlation between virulence and colony form, the " rough " varieties are generally the more virulent and are the usual forms found in pathological conditions.

Viability.—Cultures on ordinary media die in ten to fourteen days at room temperature, but survive much longer if kept at low temperatures. The organisms can be kept alive and virulent if rapidly dried at a low temperature and maintained in this state. A convenient method for preserving the viability of laboratory cultures, is to grow the organisms in a cooked-meat medium and then keep the culture at 0° C. The thermal death point is 54°–55° C.

The Streptococcus Group. — *Streptococcus hæmolyticus*, though the prevalent form of streptococcus in the commoner pyogenic infections, constitutes only one species or type of a large biological group, and comprises in itself a number of sub-species or varieties differing in certain biological characters. The streptococcus group is thus heterogeneous, and apart from the typically pathogenic forms embraces species or types which are common commensal organisms flourishing in the mouth, throat, intestines, etc. The biological classification of all these organisms presents many difficulties, and different systems of classification and nomenclature have been used at different times. Morphology, fermentation reactions, and physiological

properties, hæmolytic action and other visible effects on blood incorporated in culture medium, resistance to heat, habitat (among the commensal types), relationship to disease, aerobic or anaerobic characters, serological reactions, etc., have all been utilised in classification and nomenclature.

Morphology.—*S. hæmolyticus* shows chains of moderate length as a rule; types found in the mouth and throat vary in this respect, and long-chained forms are sometimes met with. The fæcal types (enterococcus) occur usually either as diplococci or short chains. The shape of the individual cocci varies. *S. hæmolyticus* consists usually of spheroidal forms, but among the mouth and throat types the cocci may be oval or elongated; the fæcal varieties consist of relatively large oval cocci, which may be lanceolate in appearance and resemble the pneumococcus. Capsulate varieties have been described, and the differentiation of these from the pneumococcus has sometimes presented difficulty (p 331). For practical purposes morphological differences are of little significance in the biological classification of these organisms.

Hæmolysis and other Visible Changes produced in Blood Media.—The changes produced by streptococci when growing on a medium containing blood have provided most valuable criteria for the recognition of certain main groups of these organisms. Schottmüller first employed the appearance of the colonies of streptococci on blood agar as a means of separating varieties. The medium used by him consisted of 2 parts human blood (rabbit blood may likewise be used) and 5 parts melted agar; it is, however, better to add the blood in the proportion of 5 to 10 per cent. He distinguished the "Streptococcus erysipelatis," which formed grey colonies and had a marked hæmolytic action; the "Streptococcus viridans," a short-chained organism, which produced small colonies of green colour ascribed to formation of methæmoglobin, and very little hæmolysis. Mandelbaum added to these the "Streptococcus saprophyticus," which was without hæmolytic action. Smith and Brown studying the appearances of cultures on horse blood agar, designated the co-called hæmolytic streptococci as type β, the "*viridans*" strains as type α. They regard the α type as weakly hæmolytic and draw attention to the partial hæmolysis in the discoloured zone round colonies on blood agar. A further type of reaction on blood medium has been described and designated α' type of hæmolysis; in this case there is an area of lysis round the colonies, but it is somewhat less clearly defined than the typical β hæmolysis. Discoloration is absent. The "Streptococcus saprophyticus" of Mandelbaum is Smith and Brown's type γ; it is entirely devoid of hæmolytic properties and causes no discoloration on a blood medium.

The development of a clear zone round growth on a blood agar plate has generally been taken to indicate the property of forming a diffusible hæmolysin, and among the streptococci this is usually the case; on the other hand, as emphasised by McLeod, discrepancies are sometimes noted between the results of this test for hæmolysis and those obtained by adding fluid culture to a blood suspension. For this reason McLeod recommends as a criterion of hæmolytic power the following test: 0·5 to 1·0 c.c. of a fifteen

hours' culture in 20 per cent. serum broth is added to 0·5 c.c. of a 5 per cent. suspension of washed ox blood ; the mixture is incubated for one and a half hours, and the presence or absence of hæmolysis noted. It has been generally supposed that the green coloration of *S. viridans* on blood agar is due to methæmoglobin. The colour change, however, is more marked when the organism is growing in a medium containing heated blood, and is favoured by the presence of acid. McLeod and Gordon noted that the yellow-green colour of growths of *S. viridans* and pneumococcus on heated-blood media was proportional to the amount of hydrogen peroxide which they produced, and in fact this colour change is similar to that produced artificially in the medium by means of hydrogen peroxide. It is probable, therefore, that the green coloration of heated-blood, characteristic of *S. viridans* growths, is due to an oxidation product of hæmatin (McLeod).

It is doubtful, however, whether the distinction between hæmolytic and non-hæmolytic streptococci is a fundamental character, since there is evidence that in the animal body the non-hæmolytic type may be derived from the hæmolytic. Thus, Schnitzer and Munter found after inoculation with a culture of hæmolytic streptococci that a proportion of the organisms recovered a few hours later were non-hæmolytic and reduced in virulence. According to Todd, the non-hæmolytic organism so obtained has the property of destroying the hæmolysin of *Streptococcus hæmolyticus* when the two are grown together. Fry has shown that streptococci, derived directly from the body, when grown aerobically may sometimes appear to be of the *viridans* type, and yet under anaerobic conditions yield colonies with marked rings of hæmolysis, and without any green coloration, and when tested for soluble hæmolysin give a positive result.

Physiological properties.—Fermentation reactions have been extensively used in the attempt to distinguish streptococcal types, but it has been observed by various workers that strains show considerable variability in their fermentative properties under ordinary conditions of growth and when passed through the animal body (Ainley Walker ; Beattie and Yates). Mervyn Gordon, and Andrewes and Horder originally employed a series of biochemical reactions for the differentiation of types (clotting of milk, reduction of neutral red, acid fermentation of lactose, saccharose, raffinose, salicin, coniferin, and mannitol) ; and six varieties, of which five occurred in the human subject, were recognised in this way : (1) *S. mitis* occurring normally in the saliva and fæces, (2) *S. pyogenes* the common pathogenic type, (3) *S. salivarius* another type occurring normally in the mouth, (4) *S. anginosus* found in inflammatory conditions of the throat, (5) *S. fæcalis* occurring normally in the fæces and corresponding to the organism now often designated enterococcus, (6) *S. equinus* which was found to be common in the air and dust of towns and was derived apparently from horse dung. This system of classification is only of interest now in respect of its recognition of certain outstanding types ; on the other hand, fermentative reactions have frequently been stressed as characteristic of particular varieties, *e.g.* raffinose fermentation by the salivary streptococci mannitol ; and æsculin fermentation by the fæcal streptococci. Another system of differentiating types of streptococci, based on a combination of hæmolytic

and fermentative tests (lactose, mannitol and salicin fermentation), was put forward by Holman and has been used by some workers in this subject, but must be regarded as too artificial for a satisfactory biological classification.

Recently it has been maintained that a sharp differentiation of hæmolytic streptococci from human and bovine sources respectively can be made by fermentation tests with sorbitol and trehalose, human strains fermenting trehalose but not sorbitol, bovine strains (*e.g.* from bovine mastitis) fermenting sorbitol but not trehalose (D. R. Edwards). The final acidity produced after growth in 1 per cent. glucose broth has also been said to be a distinguishing feature, the pH with bovine strains being not over 4·8, while with human strains the pH is over 5.

Various other reactions have also been employed: thus the *Streptococcus agalactiæ* (of bovine mastitis) is stated to be characteristic in its hydrolysis of sodium hippurate.

The ability to grow on a medium containing bile salt (*e.g.* MacConkey's medium) has been recognised as a distinct feature of the fæcal streptococci (enterococcus); the common pyogenic streptococci and those derived from the mouth and throat usually fail to grow in the presence of bile salt.

Resistance to heat has been a further criterion in the separation of the fæcal streptococci from other varieties (*vide infra*).

Certain streptococci differ from the common forms in their anaerobic or micro-aerophilic characters when first isolated, and some of these when growing in culture seem to exert putrefactive action.

Enterococcus.—This designation has been applied to the common type of intestinal streptococcus to which the name *S. fæcalis* was originally attached. When growing in the body, it usually occurs as a diplococcus, the individual organisms being oval or lanceolate like the pneumococcus; the members of a pair are often set at an angle and unequal in size. In cultures it shows considerable pleomorphism, and tends to grow in masses, though short chains occur in fluid media. On the surface of agar it produces a thin, semi-transparent layer with smooth margins, and there is not the tendency to form separate colonies which is shown by most streptococci. Isolated colonies are usually somewhat larger than those of other streptococci. It forms a diffuse turbidity in broth, with the formation after a time of a somewhat glairy deposit; sometimes there is a scum on the surface. It flourishes well at a lower temperature (*e.g.* 20° C.) than that at which the *Streptococcus hæmolyticus* will grow, and has great longevity in cultures. On blood agar there is no hæmolysis and no green coloration. This organism is representative of Smith and Brown's γ type (*vide supra*). Some strains, however, which correspond in other characters to the enterococcus may be definitely hæmolytic. It possesses a relatively high resistance to heat—a point of importance. Broth cultures survive exposure to 60° C. for thirty minutes, while other varieties of streptococci are killed in five to ten minutes at this temperature. When first isolated, some strains have been found to prefer anaerobic conditions. It ferments lactose, mannitol, salicin and æsculin, and coagulates milk. It differs from other types of streptococci in its ability to grow on a medium containing bile salt. Some strains have been found to liquefy gelatin.

Streptococcus lactis (or *lacticus*), the type of streptococcus found in milk and responsible for curdling, is more or less similar to the enterococcus.

Anaerobic Streptococci.—An organism of this group was first described by Veillon. Subsequent work indicates the existence of similar organisms, including different types. In morphology they are typical Gram-positive streptococci, though occurring frequently as diplococci or in short chains. They are anaerobes or micro-aerophiles when first isolated and grow best at body temperature. A suitable medium is one containing glucose and blood or serum. Some strains produce gas and putrefactive odour in culture medium. They show varied fermentation reactions like the aerobic types; hæmolytic action is not usually noticeable. In pathogenicity to animals these organisms are relatively weak, and generally prove avirulent on experimental inoculation of cultures. They have been found normally on various mucous membranes, and have been isolated from gangrenous conditions of the urogenital tract, intestine, and lungs, and in various septic lesions. Recently special attention has been drawn to their occurrence in puerperal sepsis and in this condition they may produce a general infection (*vide* p. 305).

Streptococcus agalactiæ.—This designation has been given to strains of streptococci found in the chronic form of bovine mastitis and presenting the following characters: chains long; growth in broth flocculent; milk acidified in forty-eight hours and clotted; glucose, lactose and saccharose fermented, but not mannitol; strains varying as regards hæmolysis on blood-agar including *viridans* (α), hæmolytic (β), and γ types; hydrolysing sodium hippurate. (The last-named reaction is carried out by growing the organism for five days in broth containing 1 per cent. sodium hippurate and then testing for the presence of benzoate.)

Streptococcus epidemicus.—Strains of hæmolytic streptococci possessing capsules and yielding mucoid growths on solid media have been described in milk-borne epidemics of sore-throat. Whether they merit the separate designation applied to them is doubtful. Capsule formation, though an uncommon feature of the streptococci as contrasted with the pneumococcus, must be regarded as a potential character of these organisms.

Aronson's Streptococcus.—This type is of special interest in virtue of its resemblance in certain respects to the pneumococcus (*q.v.*). As pointed out by F. Griffith it is similar to this organism in its colony structure, virulence for mice and rabbits, the formation of capsules, the production of specific precipitable substance, and the occurrence of transformation to the R form associated with attenuation in virulence. On the other hand, it differs from the pneumococcus in the round shape of the cocci, the absence of autolysis in surface colonies, insolubility in bile, the production of a hæmolysin (like that of the classical *S. hæmolyticus*) and the β hæmolysis of deep colonies in blood agar. This hæmolysin, however, is intimately associated with the organisms and is practically removed from a fluid culture by centrifuging out the streptococci (Howie). Griffith has recorded its isolation from the throat of the human subject but does not regard it as pathogenic. In the serological classification recently described by Lancefield (*vide infra*) it apparently falls into a group which comprises chiefly streptococci of bovine origin.

Streptococcus mucosus.—Non-hæmolytic streptococcal forms exhibiting capsules and yielding mucoid growths on culture medium have been described under this name. Their insolubility in bile and the absence of inulin fermentation distinguishes them from the pneumococcus though, like Aronson's streptococcus, they may constitute intermediate forms (*vide* p. 331).

Serological classification of streptococci.—Attempts have been made to classify streptococci by serological methods : agglutination with specific antisera correlated with agglutinin-absorption tests, and precipitation reactions. Agglutination tests with streptococcus cultures present some technical difficulty in view of the tendency to auto-agglutination or the granularity of suspensions prepared from the usual cultures. Growths in veal broth containing 0·2 per cent. disodium hydrogen phosphate, 0·1 per cent glucose and 1 per cent. peptone, standardised to pH 7·5 are very suitable for providing a uniform suspension. The organisms are deposited by centrifuging, the supernatant fluid is removed and a suspension of suitable density is prepared by emulsifying the deposit in saline or broth. A strain that tends to produce a granular growth in fluid medium or a granular suspension, and is therefore unsuitable for agglutination tests, may by repeated subculture in phosphate broth finally yield a uniform growth. It has been suggested, however, that as a result of this procedure a " smooth " type (as contrasted with the original " rough " form) may be selected out and the specific antigenic characters of the strain may thus be lost. The " rough " forms are generally more specific than the " smooth " varieties which tend to show " group " antigenic characters. For obtaining stable suspensions, Andrewes and Christie have recommended growing the organisms for twenty-four hours in 1 per cent. glucose broth and then inoculating legumin-agar plates from the broth cultures, the growths being washed off the plates with formol-saline. Another method which is frequently successful in yielding uniform suspensions is to centrifuge a twenty-four hours' broth culture, wash the deposited organisms three times with saline and then suspend in 0·001/N sodium hydroxide ; in the actual test the serum is diluted with M/25 sodium chloride (Smith).

Serologically the hæmolytic streptococci represent a heterogeneous group. Earlier workers on this subject attempted to classify these organisms by their agglutination reactions into well-defined types and to differentiate the scarlatinal strains from the ordinary pyogenic streptococci in this way (Dochez, Avery and Lancefield ; Bliss ; Gordon). Later work has tended to show that the hæmolytic streptococci cannot be classified

into serological types specifically associated with particular forms of infection. Strains isolated from scarlatina are themselves heterogeneous in their agglutination reactions and the same serological type may be found in different conditions. Further reference to the serology of the scarlatinal strains will be made later (p. 300). In agglutinin-absorption tests the different types show overlapping of antigenic structure; some strains possess group rather than type characters, and antigenic instability is also observed (McLachlan and Mackie). Andrewes and Christie by using graduated absorption tests found that only exceptionally were two strains serologically identical and very rarely were they entirely dissimilar. They observed a widespread occurrence of group antigens in addition to the specific constituents. Their work demonstrated the complex antigenic structure of these organisms and the difficulty of classifying them on a serological basis. Recently Griffith has been able to differentiate serologically twenty-seven types which are of epidemiological significance and has stated that specific and group serological characters respectively may be represented by different colonies in a culture (cf. *Salmonella* group, p. 542). Loewenthal has also found that different forms of colonies in a streptococcus culture may exhibit widely different serum reactions and has recognised a form of colony with highly specific characters as contrasted with a type exhibiting lesser specificity. It has been shown by Norton that the *viridans* streptococci are also serologically heterogeneous.

By precipitation tests with bacterial extracts and specific antisera Lancefield demonstrated three types of antigen among the hæmolytic streptococci: a nucleo-protein which appeared to be widely distributed among the streptococci generally, including the *viridans* group; another protein fraction which exhibited the type-specificity observed in the agglutination reactions (referred to above); and a polysaccharide which was found to be common to hæmolytic streptococci derived from human infections. It was found later that this polysaccharide was not specific for all strains of hæmolytic streptococci, which could be subdivided by precipitation tests into several groups. One of these, designated A, comprises the majority of strains which are pathogenic to the human subject. A second group (B) includes strains from bovine mastitis; but many of the strains assigned to this group are non-hæmolytic. Group C includes strains corresponding to the streptococcus of Equine Strangles (*S. equi*), and also strains from cases of bovine mastitis. It is of interest to note that one of the other groups (D) has

close affinities with the enterococcus, though the strains are apparently hæmolytic. These findings offer a possible means of distinguishing between strains which are of etiological significance in human medicine and those which are relatively non-pathogenic or are derived from animal sources.

Extracts for the precipitation test are prepared by treating growths with N/25 hydrochloric acid for ten minutes at 100° C.; the preparation is then cooled and centrifuged, the supernatant fluid is neutralised, the precipitate is discarded and the final supernatant fluid is used for the test.

While it cannot be said that a satisfactory natural classification has yet emerged from the extensive studies which have been made on this group of organisms, the following summarises broadly the characterisation of the streptococci which are commonly met with in the human subject.

A. Aerobic Types.

(1) "Hæmolytic" Streptococci; *Producing Filterable Hæmolysin.*—Chains of moderate length consisting of spheroidal cocci; viability in culture moderate; salicin usually fermented, mannitol usually not fermented; killed at 60° C. within thirty minutes; growth inhibited by bile salt; actively pathogenic as a rule to laboratory animals.

(2) "Non-hæmolytic" Streptococci; *Filterable Hæmolysin not Demonstrable in Cultures.*

(*a*) *Mouth Streptococci.*—Chains frequently long and cocci elongated; viability in culture weak; peroxide production well developed; on blood medium produce green coloration (*S. viridans*), though some types show no visible alteration of blood; frequently ferment raffinose; mannitol not fermented; killed at 60° C. within thirty minutes; growth inhibited by bile salt; virulence low.

(*b*) *Bowel Streptococci* (*Enterococcus*).—Cocci oval and in pairs or short chains; viability in culture considerable; peroxide not formed; on blood medium no visible alteration in colour; usually ferment mannitol and æsculin; resist 60° C. for thirty minutes; growth not inhibited by bile salt; produce H_2S; some liquefy gelatin; virulence low.

B. Anaerobic Types.—Distinguished by their anaerobic (or micro-aerophilic) characters; usually non-hæmolytic; virulence low.

Toxins of the Streptococci.—As stated above, many streptococci have a distinct hæmolytic action, and this is due to the production of a diffusible toxin. The amount of hæmo-

lysin formed varies greatly in the case of different strains and also according to the medium used. McLeod recommends a medium composed of 20 per cent. horse serum and 80 per cent. peptone broth with distinctly alkaline reaction. In the medium mentioned the maximum formation of hæmolysin is reached in about eighteen hours, and thereafter a diminution occurs. The hæmolysin is very labile, being destroyed at 55° C. and rapidly deteriorating, probably by oxidation, even when kept in the incubator for a few hours. The filtrate has also a toxic action on the tissues, producing focal necrosis especially in the liver of the rabbit. It is difficult to obtain antitoxin for this hæmolysin, but Todd has succeeded by using hæmolysin which had been protected against inactivation by oxidation. He has also demonstrated two different forms of lysin by cultivating the organism in serum broth and serum-free broth respectively; both were found to be equally antigenic but the antibody produced by immunising with either neutralised only the lysin of serum-free cultures. It may also be noted here that anti-streptolysin can be demonstrated in normal serum.

Among other diffusible toxic substances a leucocidin and a fibrinolytic agent have been demonstrated. Some evidence has been brought forward to show that leucocidin production can be correlated with virulence. Tillett and Garner have recently found that broth cultures and also filtrates of *S. hæmolyticus* lyse human fibrin within forty-five minutes and that serum from a patient convalescent after infection by this organism inhibits the fibrinolytic action. The significance of this property requires further investigation. The fibrinolytic agent is apparently highly stable, resisting 100° C. for sixty minutes. It is claimed that the infectivity of a strain is proportional to its fibrinolytic activity (Tillett). The scarlatinal toxin produced by strains of hæmolytic streptococci will be dealt with later. In addition to these exotoxins, substances of the nature of endotoxin have also been demonstrated in cultures of the streptococci. These will be discussed later in relation to scarlatina and acute rheumatism.

Experimental Inoculation.—The *Streptococcus hæmolyticus* is an organism the virulence of which varies much according to the diseased condition from which it has been obtained, and also one which loses its virulence rapidly in cultures. Even highly virulent cultures, if grown under ordinary conditions, in the course of time lose practically all pathogenic power. By passage from animal to animal, however, the virulence may be much

increased, and *pari passu* the effects of inoculation are correspondingly varied. Marmorek, for example, found that the virulence of a streptococcus could be enormously increased by growing it alternately (*a*) in a mixture of human blood serum and broth, and (*b*) in the body of a rabbit; ultimately, after several passages it possessed a super-virulent character, so that even an extremely minute dose introduced into the tissues of a rabbit produced acute septicæmia, with death in a few hours. It has been proved that the same species of streptococcus may produce at one time merely a passing local hyperæmia, at another a local suppuration, at another a spreading erysipelatous condition, or again a general septicæmic infection, according as its virulence is artificially increased. Such experiments are of extreme importance as explaining to some extent the great diversity of lesions in the human subject with which streptococci are associated. In addition to the general infection produced by virulent strains, localisation may occur in certain tissues, *e.g.* joints and muscles, with associated lesions, while hæmolysis and anæmia may result from the action of the toxins. Resistance of the individual host also plays a great part in determining the results of streptococcal infection.

Non-hæmolytic streptococci.—In animal experiments these types are usually of low virulence. After intravenous injection they show a tendency to localise on the endocardium, producing endocarditis with vegetations. Some workers have found also a selective localisation in synovial membranes, the pericardium, kidney, and in the cerebro-spinal fluid. Such results have suggested the relation of organisms of this type to acute rheumatism and its complications (*vide infra*). Localisation in the gall-bladder with resulting cholecystitis and calculus formation has also been recorded in the case of streptococci isolated from the gall-bladder and cystic lymph gland of cases of cholecystitis in the human subject.

Lesions in the Human Subject.—*Hæmolytic streptococci* are especially found in spreading inflammation with or without suppuration, in diffuse phlegmonous and erysipelatous conditions (*vide infra*), suppuration in serous membranes and in joints. They are common in wound suppurations, usually along with other pyogenic organisms. It should be noted that in certain cases of post-operative wound infections by hæmolytic streptococci the source of the organisms may possibly have been the mouth or nose of other persons in the proximity of the operation area. For this reason, masking of the mouth and nose has become a routine procedure among those in immediate

attendance at a major surgical operation. During the War streptococci were usually to be found in gun-shot wounds; at first the enterococcus abounded, at a later stage hæmolytic streptococci were the more common type. They also occur in acute suppurative periostitis and ulcerative endocarditis (*vide infra*). Lymphangitis and secondary abscesses in lymphatic glands are also most frequently caused by streptococci. These lesions often occur very quickly when a virulent streptococcus has gained entrance through a prick or scratch in the skin, and in such cases there may be little or no inflammatory reaction at the site of entry of the infection. Impetigo is generally attributed to these organisms. They produce fibrinous exudation on the mucous surfaces, leading to the formation of false membrane, *e.g.* in the throat in scarlatina and other conditions, and they are also the organisms most frequently present in acute catarrhal inflammations in this situation. The relation of streptococci to scarlatina will be discussed in detail later. Epidemics of sore throat, often of a severe type, have been attributed to streptococci spread by milk and derived from udder infection in the cow. Suppurative otitis media with its sequelæ—*e.g.* mastoiditis, meningitis—is frequently streptococcal in origin. Both hæmolytic and non-hæmolytic streptococci are found in the broncho-pneumonic conditions occurring as complications of other infections, *e.g.* influenza. In puerperal sepsis they are frequently found in pure or mixed infection, and they appear to be the most frequent cause of puerperal septicæmia (*vide infra*). In a certain proportion of cases they also produce peritonitis secondary to appendicitis. In pyæmia they are frequently present, in some cases associated with other pyogenic organisms.

Non-hæmolytic streptococci.—All are generally of lower virulence and associated with less acute lesions. They are slowly invasive and produce a low type of inflammation in which suppuration and tissue destruction are not marked. They are frequent in tonsillitis, otitis, dental abscess, pyorrhœa alveolaris, and bronchitis. They may occur, however, in broncho-pneumonia and even in septicæmic conditions. Non-hæmolytic streptococci, both of the *viridans* and enterococcus types, are associated with a certain proportion of cases of cholecystitis and can be isolated from the bile, gall-bladder wall, or cystic lymph gland. The condition of subacute infective endocarditis (*vide infra*) is due to this type of organism. The question of their association with acute rheumatism is referred to later. Some cases of enteritis in infants—streptococcal enteritis—have been attributed to a

streptococcus, which is usually of the enterococcus type. The lesions in which the enterococcus is found are mainly those where infection can be traced from the bowel, *e.g.* cystitis, appendicitis, cholecystitis. It has been found also in abscesses following typhoid fever. It is difficult, however, to assess the pathogenicity of this organism.

Scarlatina.—It has been long recognised that streptococci are usually present in large numbers in the throat in scarlatina, and that many of the complications of this disease are undoubtedly streptococcal. At one time, streptococci were regarded, in virtue of their occurrence in the disease, as the likely etiological agent, and the associated strains were designated *Streptococcus scarlatinæ*. Thus, in 1887 Klein as a result of the investigation of milk-borne outbreaks, claimed that the disease was due to a definite type of streptococcus, but subsequent work showed that the scarlatina streptococcus was similar in its cultural characters to the pyogenic strains and had no specific pathogenic effects on animals. Later views tended to reverse the original idea, although the almost constant occurrence of hæmolytic streptococci in the throat was well recognised, and it was supposed that these organisms represented a secondary infection and that the primary etiological agent was a different and undetermined type of micro-organism possibly belonging to the category of the filterable viruses. Attempts to demonstrate a filterable virus of scarlatina, however, have led to negative or inconclusive results.

In 1923, G. F. and G. H. Dick were able to produce scarlet fever in the human subject by infecting the throat with a culture of a hæmolytic streptococcus isolated from a known case of the disease (a finding which has been confirmed by subsequent workers), and in 1924 they demonstrated that the scarlatina streptococcus produces a diffusible " toxin," present in filtrates from cultures, which, injected intracutaneously in persons susceptible to the disease, elicits a reaction of cutaneous erythema and inflammation ; while non-susceptibles (*e.g.* convalescents) fail to show the reaction. This reaction is therefore analogous to the Schick reaction for susceptibility to diphtheria (*q.v.*), and is now designated the Dick reaction. They also produced symptoms of scarlet fever by injecting subcutaneously the filtrate of a culture of the scarlatina streptococcus. The validity of the Dick reaction has been generally accepted. Thus persons during the early stage of the disease (first three days), in the majority of cases, exhibit a positive reaction illustrating apparently their susceptibility to the " toxin " and the absence of any natural or

acquired immunity. On the other hand, convalescents usually show a negative reaction which suggests the acquisition of an antitoxic immunity as a result of the infection. Discrepancies, however, are sometimes observed between the results of the test and the clinical state (*vide infra*). It has also been shown by the so-called Schultz-Charlton reaction that the serum of convalescents contains a neutralising antitoxin, *i.e.* the intracutaneous injection of the convalescent serum (*e.g.* 0·2 c.c. of a 1 in 10 dilution) produces a local blanching or "extinction" of the rash in an active case. Further, the serum of convalescents neutralises the Dick toxin when mixed with it and injected into the skin of a known susceptible person. These facts, together with the constant occurrence of hæmolytic streptococci in the throat, strongly support the view that scarlatina is due to infection of the throat with a hæmolytic streptococcus capable of producing a specific diffusible toxin, which is responsible for the general manifestations of the disease, and that the infection is in the first instance local, the general condition being essentially a toxæmia.

The question arose whether the streptococcus of scarlatina constitutes a specific type. This led to extensive studies of the serological characters of strains of hæmolytic streptococci from scarlatina and other conditions. The results at first suggested that the scarlatina streptococci might be classified in certain serological groups, but later observations have tended to show that there is no line of demarcation serologically between the scarlatina strains and other hæmolytic streptococci (McLachlan and Mackie). The serology of the hæmolytic streptococci has already been discussed (p. 293) and it has been seen that strains from different sources may belong to the same serological type. It has also been shown that hæmolytic streptococci from cases of erysipelas, infected wounds, and normal throats, may produce toxins similar to that obtained from known scarlet fever strains.

According to Griffith a majority (over 60 per cent.) of scarlatinal strains of hæmolytic streptococci fall into four serological types while the remainder are serologically heterogeneous and individualistic. Though these four types are frequently represented among scarlatinal strains they do not invariably predominate and in particular outbreaks a majority of the strains may be serologically different from Griffith's types. Practically all scarlatinal streptococci can be assigned by precipitation reactions to Lancefield's A group (*vide* p. 294).

An obstacle to the experimental study of the etiology of the disease is the fact that laboratory animals are generally insus-

ceptible to the toxin. The only certain criterion, therefore, of the identity of a scarlatina-producing streptococcus depends on whether culture-filtrates in suitable dilution yield a positive Dick reaction in known susceptibles (*e.g.* early scarlet fever cases) and negative reactions in known negative reactors (*e.g.* convalescents). The neutralisation by an antitoxic serum (*vide infra*) of the reacting property of the filtrate constitutes a confirmatory test.

Though animals are usually insusceptible to the toxin even in large doses, it has been stated that young goats give a cutaneous reaction analogous to the Dick reaction (Kirkbride and Wheeler), and the same has been noted among chinchilla rabbits (Fraser and Plummer). Concentrated toxins prepared by Hartley and by Pulvertaft have proved lethal in rabbits Trask has also found that some rabbits exhibit susceptibility to the scarlatinal toxin, which is neutralisable by the specific antitoxin, and that susceptibility tends to increase with the age of the animal. Duval and Hibbert have reported that a culture-lysate is more potent than a filtrate, and they have produced marked toxic effects, including acute hæmorrhagic nephritis, in dogs, by means of such lysates

The logical outcome of recent work on scarlet fever has been (1) the active immunisation with toxin for the prophylaxis of the disease, as in active immunisation against diphtheria (*q.v.*), and (2) the treatment of the disease by passive immunisation with antitoxic sera. It has been shown that by immunisation of horses with the toxin, a neutralising antiserum can be obtained, and such sera are now being utilised successfully in treatment.

The Dick reaction has shown that the age of maximum susceptibility to scarlet fever is between six months and five years. Over twenty years of age about 80 per cent. of persons are immune.

While the diffusible principle of the scarlatinal streptococci, which is now spoken of as " toxin," has been commonly regarded as analogous to other bacterial exotoxins, it has been suggested that the scarlet fever rash may be a phenomenon of supersensitiveness to the proteins of the streptococcus, and that the Dick reaction is an index of such sensitiveness (*vide* Chapter VI.). This supersensitiveness is supposed to be the result of preexisting subinfection. Guinea-pigs which normally fail to react to the Dick toxin can apparently be sensitised by injection of filtrates or cultures so that a subsequent intracutaneous injection produces a reaction similar to the Dick reaction. This reaction, however, is not strictly analogous to the Dick reaction in the human subject (Mackie and McLachlan).

It has been shown that culture-filtrates as ordinarily used contain two reacting substances: a true exotoxin which is labile at 80° C. and is neutralised by scarlatinal antitoxin; and a nucleo-protein which is stable up to 100° C., though partially inactivated after half an hour at this temperature (Ando, Kurauchi and Nishimura). The usual Dick reaction is stated to be due to the presence of both substances, the reaction to the protein being allergic in nature. It has been customary in carrying out the control test for the Dick reaction to use a preparation heated to 100° C. for at least one hour in view of the difficulty of inactivating the material. This difficulty may be due to the reactive nucleo-protein referred to above. It has also been noted that during the course of the scarlatinal illness patients may develop a reactiveness (on cutaneous testing) to the dissolved intracellular products of hæmolytic streptococci, indicating the acquisition of allergy at a time when they are becoming immune to the streptococcal exotoxin (Gibson and McGibbon). These observations serve to explain confusing results obtained at times in the application of the reaction, and a purified exotoxin free from bacterial protein has been advocated for the test.

To sum up, all recent work on scarlatina has clearly demonstrated the important relationship of hæmolytic streptococci to the disease, and throws light on the genesis of a disease for long regarded as etiologically obscure. An explanation is also afforded of the occurrence of the so-called "Surgical scarlatina," the result of a streptococcal wound infection. "Puerperal scarlatina" can be similarly explained.

THE DICK REACTION: *Preparation of toxin.*—A suitable strain of "*Streptococcus scarlatinæ*" is grown at 37° C. for two days in Hartley's broth (pH 7·5). The culture is centrifuged at high speed, and the supernatant fluid removed and filtered through a tested Berkefeld filter at low pressure. The filtrate constitutes the "toxin"; 0·5 per cent. phenol should be added for preservation. In the test a suitable dilution is used, *e.g.* 1 : 1000 in normal saline or preferably a buffered salt solution as in the Schick test (*q.v.*), and 0·2 c.c. is injected intracutaneously, usually on the forearm. A control test should be carried out simultaneously with the same amount of toxin after heating at 100° C. for one hour.

Toxin preparations are standardised according to the "skin-test dose," *i.e.* the least quantity of toxin which when injected intradermally in susceptible persons produces within twenty-

four hours a reaction of 1 cm. or more in diameter. The dilution used for the routine Dick test should be such that 0·2 c.c. contains one skin-test dose. It is doubtful, however, whether the methods employed at the present time for the standardisation of this toxin are sufficiently exact.

The positive reaction develops in six to twelve hours, and appears as a bright scarlet erythematous reaction often 30 mm in diameter. Its maximum is usually attained within twenty-four hours, and it then gradually fades and has practically disappeared by the third day. Readings should be made not later than twenty-four hours. Usually the control test leads to no definite reaction, but occasionally pseudo-reactions of varying degree are met with.

A purified preparation of the toxin may be obtained by the following method: the filtrate is mixed with 3 volumes of absolute alcohol and after thirty minutes' standing the precipitate is removed by centrifuging; this is dissolved in normal saline (1/10th of original volume of filtrate); the pH is adjusted to 4·0–4·2 by adding 33 per cent. acetic acid and the preparation is allowed to stand overnight when it is centrifuged and the precipitate discarded; the supernatant fluid is heated for thirty minutes with 3 volumes of absolute alcohol and the precipitate removed by centrifuging and dissolved in a volume of normal saline equal to 1/10th the volume of the original material dealt with; the pH of the solution is finally adjusted to 7·0 by the addition of decinormal sodium hydrate.

Active Immunisation is carried out by the subcutaneous injection of graded quantities of toxin. A series of five injections are given at weekly intervals as follows: (1) 500, (2) 2500, (3) 20,000, (4) 40,000, (5) 80,000–100,000 skin-test doses. Two to four weeks after the last injection the Dick test is repeated to ensure that immunisation has been effective.

Passive Immunisation and Serum Therapy.—For this purpose the antitoxic serum referred to above is used. It is obtained by immunising horses with graduated doses of toxin over a period of several months. The serum is refined by the usual methods. The antitoxin is usually standardised according to its potency in neutralising the toxin. The antitoxic unit adopted in the United States is the smallest amount of antitoxin which neutralises 50 skin-test doses of the scarlatinal streptococcus toxin, the quantitative neutralisation tests being carried out in human reactors. It is doubtful whether this system is sufficiently accurate to yield exact numerical values. O'Brien and his co-workers have claimed that fairly accurate standardisation can be obtained by quantitative protective tests in rabbits: the animals are injected intravenously with serum and after four to six hours with a virulent streptococcus culture; by varying the dosage the protective value of different sera can be

compared. Another method consists in adding a constant volume of varying dilutions of antitoxin to a fixed dose of toxin, heating the mixtures at 50° C. for two hours and then injecting 0·05 c.c. of each intradermally into rabbits, which are kept at 30° C.; the reactions are rendered more local and the readings thereby improved if the animals receive a small intravenous dose of anti-scarlatinal serum on the previous day (Buttle and Lowdon). The initial therapeutic dose in terms of the American standard should be 6000 units, but in severe cases larger and repeated doses may be required. Administered intravenously in the early stage of the disease, the serum exerts a definite therapeutic effect, lessening the toxic manifestations, shortening the illness and reducing the incidence of complications.

Erysipelas.—A spreading inflammatory condition of the skin may be produced by a variety of organisms, but the disease in the human subject in its characteristic form is almost invariably due to a streptococcus, as was shown by Fehleisen in 1884. He obtained pure cultures of the organism, and gave it the name "Streptococcus erysipelatis," and, further, by inoculations in the human subject as a therapeutic measure in malignant disease, he was able to reproduce erysipelas. There is, however, no essential difference between the streptococcus of erysipelas and the hæmolytic streptococci of suppuration. It must be noted, however, that erysipelas may be transmitted from one person to another, and purulent conditions due to streptococci do not appear liable to be followed by erysipelas. On the other hand, the connection between erysipelas and puerperal septicæmia is well established clinically. Recent studies of streptococci from cases of erysipelas with particular reference to their toxins and serological reactions have elicited no clear distinction between these strains and hæmolytic streptococci from other sources.

In a case of erysipelas the streptococci are found in large numbers in the lymphatics of the cutis and underlying tissues, just beyond the swollen margin of the inflammatory area. As the inflammation advances they gradually die out, and after a time their extension at the periphery comes to an end. The streptococci may extend to serous and synovial cavities and set up inflammatory or suppurative change—peritonitis, meningitis, and synovitis may thus be produced.

Puerperal Sepsis.—While other pyogenic organisms are frequently associated with puerperal sepsis, *e.g.* staphylococci or *B. coli*, the streptococci represent the most prevalent and probably the most formidable type of organism responsible for

such infection. Though only localised inflammatory and suppurative lesions may result, general septicæmia is not infrequent, and then the infection is generalised almost from the outset and presents a clinical and pathological picture of extreme severity. Puerperal sepsis is in most cases due to an exogenous infection with virulent strains of *Streptococcus hæmolyticus*. It is, of course, possible that in some cases infection may be endogenous, the organisms entering the uterus or being introduced mechanically from the lower genital passages, and it has been shown that non-hæmolytic and even hæmolytic streptococci occur in the vagina in pregnant women. However, the virulence of such vaginal strains is doubtful. Thus, by means of precipitation tests Lancefield and Hare have found that the majority of hæmolytic streptococci occurring in the birth-canal of pregnant women who remain afebrile in the puerperium do not belong to Lancefield's Group A, *i.e.* the strains which are pathogenic to the human subject (*vide* p. 294). They appear to correspond serologically with the hæmolytic streptococci occurring in bovine mastitis or with strains which are possibly of fæcal origin. Though non-hæmolytic strains have been found in some cases of the disease. the most frequent organism in puerperal infections, and that which causes the most serious results, is the *Streptococcus hæmolyticus*. There is now a considerable amount of clinical and bacteriological evidence that infection may be transmitted from medical and nursing attendants who are harbouring a virulent strain of this organism, *e.g.* in the throat. Smith has pointed out in a series of cases that the strain isolated from the patient is serologically identical with a hæmolytic streptococcus found in the attendant. Of course , when once a case of puerperal sepsis has developed there is every likelihood that the infection will be conveyed by the hands of attendants, etc., to other parturient women unless the strictest precautions are taken. Recently Colebrook has drawn special attention to the frequent occurrence of anaerobic streptococci (demonstrable by anaerobic blood culture) in cases of puerperal fever occurring in a large maternity hospital. The characters of the anaerobic streptococci have already been referred to (p. 292). It seems that such infections are probably endogenous in origin.

Acute Rheumatism.—There are many facts which indicate the infective nature of this disease, and investigations from this point of view have yielded important results. A type of streptococcus was originally isolated from cases of rheumatism by Triboulet, and by Westphal and Wassermann, and it was

first studied in this country by Poynton and Paine. It was regarded as a specific organism and named "*Micrococcus rheumaticus*." It is a Gram-positive streptococcus growing in pairs or short chains; fermenting various sugars, including mannitol, with acid production, and usually clotting milk after incubation for two days; non-hæmolytic on blood agar; growing well on gelatin at 20° C. Injection of pure cultures in rabbits often produced polyarthritis and synovitis, valvulitis and pericarditis, without any suppurative change. In one or two instances choreiform movements were observed after injection. The organism was reported to be most easily obtained from the substance of inflamed synovial membrane. It was only occasionally obtained from the fluid in joints. It was also cultivated from the blood in rheumatic fever, from the vegetations on the heart valves, and from other acute lesions; in many cases, however, cultures from the blood gave negative results. Poynton and Paine cultivated this type of streptococcus from the cerebro-spinal fluid in three cases where chorea was present, and also detected it in the membranes of the brain. They considered that this disease is probably of the nature of a slight meningo-myelitis produced by the organism. The "*Micrococcus rheumaticus*" described by the earlier workers does not represent a specific type, and non-hæmolytic streptococci with somewhat varying biological features, *e.g.* fermentative reactions, have been isolated from the disease by different workers. Many of the strains described have been *viridans* streptococci, but a number of "γ" type strains have also been found. In evaluating these findings, it must be remembered that streptococci from the throat and intestines may enter the blood and localise in damaged tissues, so that the presence of these organisms in exudates and synovial membranes in acute rheumatism is not necessarily significant from the etiological standpoint. Birkhaug and others have described a form of "γ" type streptococcus with distinctive fermentative reactions—fermenting saccharose, salicin, raffinose, inulin, and usually lactose, but not acting on mannitol. Many of the strains of this type, however, have been isolated from the throats of rheumatic cases, and differ from strains which have been found by other workers in the blood and tissues. It must be emphasised that the finding of streptococci is by no means uniform, and frequently no organisms can be cultivated from the blood, exudates, or tissues. Some observers have maintained that an exotoxin formed by the infecting streptococcus may be responsible for the manifestation of the disease, the organism remaining

localised, *e.g.* in the throat; and Birkhaug and others claim to have demonstrated such toxin in culture filtrates by a cutaneous reaction analogous to the Dick reaction of scarlatina. This has not been confirmed. It must be noted that the lesions produced in animals by inoculation with streptococci of rheumatic origin may also result from the intravenous injection of other non-hæmolytic streptococci, *e.g.* those isolated from the mouth and throat. This is particularly true in regard to the endocardial lesions, and the relationship of streptococci to endocarditis will be referred to later.

It has also been suggested that the phenomena of the disease depend on supersensitiveness to the products of streptococci (Zinsser and Yu, and others). According to this theory, previous streptococcal infection may sensitise the tissues, with the result that subsequent infections lead to allergic effects which constitute the general manifestations of the disease. Recently evidence has been brought forward by Coburn and others that *S. hæmolyticus* may be the causal organism in acute rheumatism. The site of the primary infection is in the upper part of the respiratory tract, and there occurs an attack, *e.g.* of sore throat, which is recovered from; then after a " silent " period, which usually lasts from ten to twenty days, the acute rheumatic illness develops. As regards the mechanism of its production, the streptococcus cannot be recovered from the blood, although it may localise in the spleen and pericardial lymph glands (Collis). Accordingly, it has been suggested that acute rheumatism is due to a reaction of the tissues to products of *S. hæmolyticus* in specially predisposed individuals. In support of this view is the observation that in rheumatic subjects an intradermal injection of nucleoproteins of the streptococci produces in a high proportion of cases marked local inflammatory reactions, which sometimes resemble the cutaneous manifestations of rheumatism. Collis has obtained similar results by the injection of a soluble " endotoxin " prepared by extracting with saline the ground-up bodies of the streptococci which had previously been killed by heating at 60° C.; he finds that this substance is much more resistant to heat than the Dick toxin (*q.v.*). It is of interest in this connection that Todd has demonstrated a specific antitoxin for streptolysin in the serum of a high proportion of acute rheumatic cases. A number of instances have also been reported in which acute rheumatism has occurred in outbreaks and it has been suggested by Glover that the disease may be spread by droplet infection from the upper respiratory passages.

The etiological relation of streptococci to the disease has recently been investigated in Edinburgh by Gibson and Thomson who have examined a considerable number of acute rheumatic cases as regards clinical history, antecedent tonsillitis, scarlatina and other infections, the presence of hæmolytic streptococci in the throat and the dermal reaction to extracts of hæmolytic streptococci. One definite fact emerged from their inquiry, namely, the association between infection by hæmolytic streptococci and the rheumatic affection, but they concluded that this was not a simple matter of cause and effect and postulated some other etiological factor. Thus the dermal reaction did not appear to be significantly more frequent in the rheumatic group than in a control non-rheumatic series. In fact, this reaction appeared to indicate merely a previous infection with hæmolytic streptococci. They suggested that acute rheumatism may be due to some infective agent not yet defined whose entry into the body is facilitated by streptococcal infection, or alternatively that the allergic manifestations of this infection may predispose certain tissues to an unknown virus or facilitate its generalisation. Only in this way can the close epidemiological relationship of hæmolytic streptococci to the disease be reconciled with the widespread distribution of this streptococcal infection and the comparatively limited incidence of acute rheumatism. Goldie and Griffiths have recently reported an extensive investigation of acute and chronic rheumatic cases, and, taking as evidence of previous infection by hæmolytic streptococci specific serological and allergic reactions, have concluded that such infection is more common in rheumatic patients than in healthy persons otherwise comparable.

The possibility that a filterable virus may be present in acute rheumatism will be discussed in Chapter XXV.

Streptococcal Food Poisoning.—It has been recently reported by Jordan and Burrows that poisoning may result from the ingestion of food contaminated by streptococci, as in the case of foods in which staphylococci have been growing and producing toxin (*vide* p. 282).

Immunity to Streptococcal Infection.—Immunity following recovery from a streptococcal infection is often quite transitory, as in the case of erysipelas, so that subsequent or repeated attacks of the infection may occur. The antitoxic immunity following scarlatina on the other hand is more lasting. Animals can be immunised against these organisms by the injection of graded doses of cultures (see Chapter VI., p. 222). The serum

acquires protective properties and can confer passive immunity. *In vitro* it also exhibits various antibody reactions, *e.g.* opsonic, agglutinating, precipitating, but possesses no immune bactericidal action. Immunity produced by a particular strain is often extremely restricted as regards other strains in virtue of the immunological heterogeneity of the group, as has been described above (p. 293). Immunisation with culture-filtrates of the hæmolytic streptococci leads to an antitoxic immunity ; there is no evidence of heterogeneity in the toxins produced by different strains. Under experimental conditions, an antitoxic serum may protect animals from the lethal effect of virulent streptococci which have been injected intravenously. A polyvalent antistreptococcus serum has frequently been utilised for the treatment of acute infections but generally the results have been disappointing. The use of an antitoxic serum in the treatment of scarlatina has already been dealt with (p 303.) and it may be noted that this type of antiserum has also been applied with some success in other infections due to hæmolytic streptococci in which severe toxic manifestations occur, *e.g.* erysipelas, etc. The question of local immunity to streptococcal infection is discussed in Chapter VI., p. 209. Vaccine therapy is dealt with later (p. 323).

Other Pyogenic Infections

Endocarditis.—In the *simple type* of acute endocarditis, so often the result of acute rheumatism, the presence of micro-organisms in the affected tissue and vegetations has only rarely been demonstrated. Certain observers have in the past described the occurrence of streptococci of the non-hæmolytic type which they have designated "*SM. rheumaticus*," and the biological characters of these have already been referred to (p. 305). It is exceedingly doubtful, however, whether rheumatic endocarditis is due to actual localisation of streptococci in the endocardium. It is nevertheless a well-established fact that non-hæmolytic streptococci isolated from various sources may on intravenous injection in rabbits produce endocarditis with vegetations on the valves; the organisms under such conditions localise in the endocardium and are found in large numbers in the associated vegetations. Such experimental endocarditis more closely resembles the condition described as *subacute infective endocarditis* in the human subject. This condition has come to be recognised as almost a specific disease and is nearly always due to a streptococcus of the *viridans* or entero-

coccus type. The lesion is probably the result of localisation of streptococci in the endocardium from the circulating blood, a congenital abnormality of the valve or pre-existing damage due to rheumatic disease being the main determining factors. This presupposes the occurrence in such cases of an initial streptococcal bacteriæmia from some focus of infection and the recent observations of Okell and Elliott have shown how transient

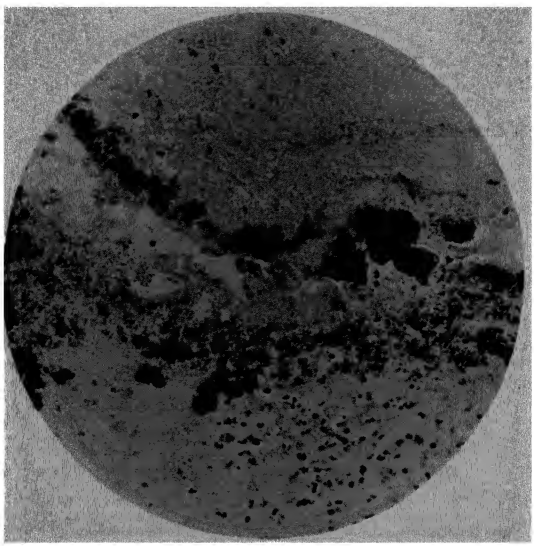


FIG. 32 —Section of a vegetation in ulcerative endocarditis, showing numerous pneumococci lying in the spaces. Stained by carmalum and Gram's method. ×300.

bacteriæmia by non-hæmolytic streptococci may be associated with oral sepsis particularly following extraction of teeth. The disease is one of considerable duration and is almost invariably fatal. The left side of the heart is most frequently affected and large chronic vegetations develop with little or no tissue destruction. Embolism may result. There is marked splenic enlargement associated with the condition and frequently a glomerular nephritis. Bacteriæmia is frequent but not invariable. The organisms are easily demonstrable in the vegetations. This condition would appear to be intermediate between the simple endocarditis and the *acute bacterial, ulcerative,* or

malignant type in which there is marked destruction of tissue. This third type of endocarditis may be produced by various organisms, mainly of the pyogenic category. The most destructive form of the disease is due to hæmolytic streptococci and staphylococci, the former being the more frequent. In some cases following pneumonia the pneumococcus (*q.v.*) is present (Fig. 32); in these the vegetations often reach a large

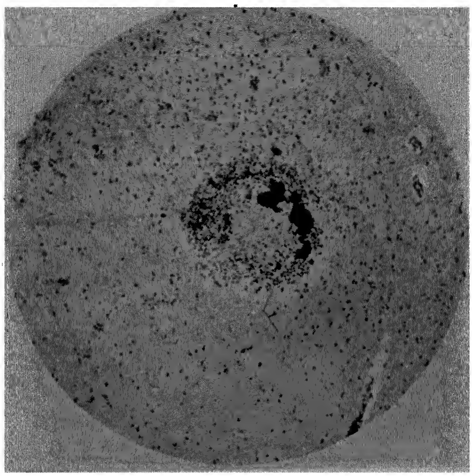


Fig. 33.—Minute focus of commencing suppuration in brain—case of acute ulcerative endocarditis. In the centre a small hæmorrhage; to right side dark masses of staphylococci; zone of leucocytes at periphery.

Carmalum and Gram's method. ×50.

size and have not the same tendency to break down as in the staphylococcal infections, where infective emboli tend to give rise to metastatic abscesses (Figs. 33, 34). In some instances *Bacillus coli* has been found, and occasionally in endocarditis following typhoid fever *Bacillus typhosus* has been described as the organism present. The meningococcus and the gonococcus have also been reported in endocarditis, but such occurrences are relatively rare. Tubercle nodules in the valves have been found in a few cases of tuberculosis, though no ulcerative condition is usually produced.

Experimental.—Occasionally ulcerative endocarditis is produced by the simple procedure of injecting staphylococci or streptococci into the circulation. It often follows, however, when the valves

have been previously injured. Orth and Wyssokowitsch at a comparatively early date produced the condition by damaging the aortic cusps by a glass rod introduced through the carotid, and afterwards injecting staphylococci into the circulation. Similar experiments have since been repeated with streptococci, pneumococcus, and other organisms, with like result. Ribbert found that if a potato culture of *Staphylococcus aureus* were rubbed down in salt solution so as to form an emulsion, and then injected into

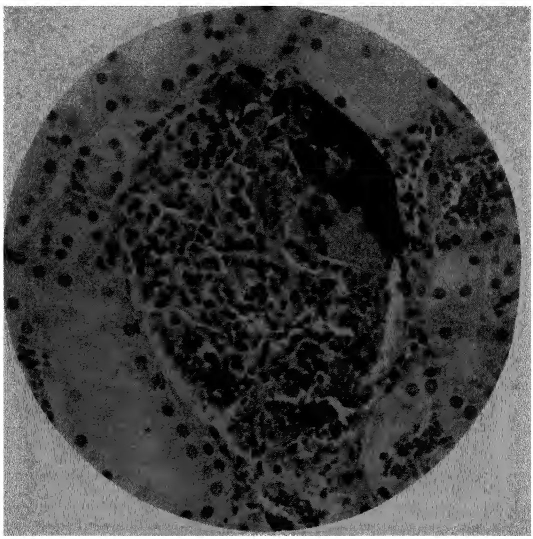


FIG. 31.—Secondary infection of a glomerulus of kidney by the *Staphylococcus aureus*, in a case of ulcerative endocarditis. The cocci (stained darkly) are seen plugging the capillaries and also lying free. The glomerulus is much swollen, infiltrated by leucocytes, and partly necrosed.
Stained by carmalum and Gram's method. ×250.

the circulation, some minute fragments became arrested at the attachment of the chordæ tendineæ and produced an ulcerative endocarditis.

Acute Suppurative Periostitis and Osteomyelitis.—Special mention is made of this condition on account of its comparative frequency and gravity. The great majority of cases are caused by the pyogenic cocci, of which one or two varieties may be present, *Staphylococcus aureus*, however, occurring most frequently. The pneumococcus has been found alone in some cases, and in a considerable number of cases following typhoid fever

Bacillus typhosus has been found alone. In others, again, *Bacillus coli* is present.

The infection of the periosteum or interior of the bones by these organisms, which is especially common in young subjects, may take place in the course of other infections produced by the same organisms or in the course of infective fevers, but in a great many cases the path of entrance cannot be determined. In the course of this disease serious secondary infections are always very liable to follow, such as small abscesses in the kidneys, heart wall, lungs, liver, etc., suppuration in serous cavities, and ulcerative endocarditis ; in fact, some cases present the most typical examples of extreme general staphylococcal infection. The entrance of the organisms into the blood stream from the lesion of the bone is especially favoured by the arrangement of the veins in the bone and marrow.

Experimental.—Multiple abscesses in the bones and under the periosteum may follow simple intravenous injection of the pyogenic cocci, and are especially liable to be formed when young animals are used These abscesses are of small size, and the processes do not spread in the same way as in the natural disease in the human subject

In experiments on healthy animals, however, the conditions are not analogous to those of the natural disease We must presume that in the latter there is some local susceptibility, which enables the few organisms which have reached the part by the blood to settle and multiply. Moreover, if a bone be experimentally injured, *e g.* by actual fracture or by stripping off the periosteum before the organisms are injected, then a much more extensive suppuration occurs at the injured part.

Focal Infection.—This term is commonly applied in clinical medicine and surgery to chronic localised inflammatory or suppurative lesions from which bacteria may enter the blood stream and then localise in some other part of the body or from which bacterial products are absorbed and produce lesions in other parts either by direct toxic action or as a result of an allergic state. Such chronic focal infections are not uncommon in various situations, *e.g.*, teeth, gums, tonsils, nasal sinuses, uterus, etc. Streptococci are frequently the associated organism and a variety of conditions of doubtful etiology has been attributed to this cause, *e.g.* rheumatoid arthritis, chronic rheumatism, cholecystitis, pancreatitis, irido-cyclitis, gastric and duodenal ulcer. The evidence on this question is quite inconclusive though undoubtedly systemic effects and remote lesions may result from such chronic septic foci.

Acne.—In the pus of acne lesions and also in the comedones a bacillus, now generally known as the *Acne bacillus* (*Corynebac-*

terium acnes), may be found in large numbers. The organism, which may now be regarded as one of the diphtheroid group (*vide* p. 396), was first described by Unna and afterwards cultivated by Sabouraud. It occurs in the form of short rods, sometimes swollen at one end, and measuring about 2 μ in length and rather less than 0·5 μ in thickness. It often shows a beaded appearance like that of a diphtheroid bacillus. It stains readily with the basic aniline dyes and retains the stain in Gram's method. It grows best in the presence of only a small quantity of atmospheric oxygen, being a micro-aerophilic organism. In shake or stab cultures, growth is at a maximum in a limited zone about half an inch from the surface. It can be cultivated by the usual anaerobic methods. Glucose agar is a suitable medium. In such a medium after three or four days' incubation at 37° C. small whitish colonies appear, which when examined under a low magnification are seen to have a lenticulate shape. It can also be cultivated aerobically on a serum or blood medium with an acid reaction (pH 6·2–6·8). The organism shows considerable pleomorphism—coccoid, diphtheroid, and filamentous types being present, as well as irregular bizarre forms. Some observers have also obtained surface growth on ordinary agar, especially after the organism has been cultivated for some time under anaerobic conditions. Its relation to the suppuration in acne has been a matter of dispute, some holding that it is the cause of the suppuration, whilst others maintain that this is due to pyogenic cocci.

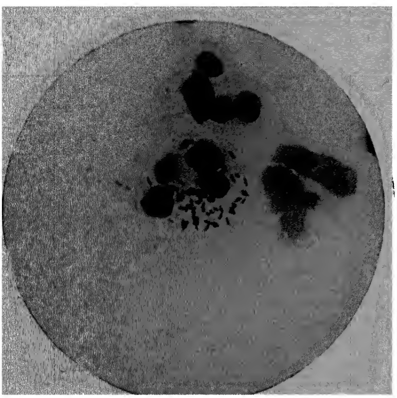


FIG. 35.—Film preparation from a case of acute conjunctivitis, showing Koch-Weeks bacilli, chiefly contained within a leucocyte. (From a preparation by Dr. Inglis Pollock.) ×1000.

Conjunctivitis.—A considerable number of organisms are concerned in the production of conjunctivitis and its associated lesions. Of these, certain appear to be specially associated with this region. Thus an organism generally known as the Koch-Weeks bacillus (*Hæmophilus conjunctivitidis*) is the most common cause of acute contagious conjunctivitis, especially prevalent in Egypt,

but also occurring in this country. It can readily be found in the muco-purulent secretion by staining films with weak carbol-fuchsin, and is often to be seen in the interior of leucocytes (Fig. 35). This organism is very minute, being little more than 1 μ in length, Gram-negative, and morphologically resembles the influenza bacillus (Fig. 36). Its conditions of growth are practically the same as those of *B. influenzæ* (*q.v.*); thus it flourishes on a medium containing blood but will not grow on ordinary media, and may be classified with the group of "hæmophilic" bacteria. Some observers regard it as biologically

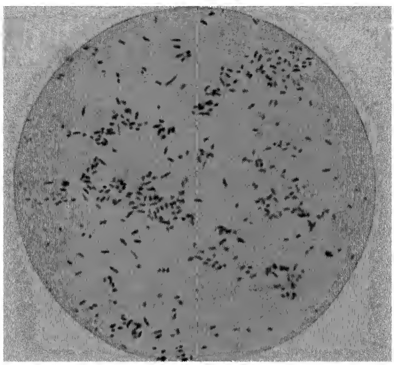


FIG. 36.—Koch-Weeks bacillus, from a young culture on blood agar. Stained with weak carbol-fuchsin. ×1000.

identical with *B. influenzæ*. On blood agar it produces minute transparent colonies like drops of dew. The obtaining of pure cultures is a matter of considerable difficulty, and it is nearly always accompanied by the *Bacillus xerosis*. The organism described in the literature as "Müller's bacillus" is apparently a closely allied type.

Another organism which causes conjunctivitis is the diplo-bacillus first described by Morax; it is often known as the Morax-Axenfeld bacillus (*Hæmophilus lacunatus*). It is especially common in the more subacute cases of conjunctivitis. Eyre found it in 2·5 per cent. of all cases of conjunctivitis. This organism is a small plump bacillus, measuring 2×1 μ and usually occurring in pairs, or in short chains of pairs (Fig. 37). It is non-motile, does not form spores, and is decolorised by Gram's method. It does not grow on the ordinary media, the addition of blood or serum being necessary. Its optimum tempera-

ture is 37° C., and it does not flourish at room temperature. On solidified serum it forms small rounded colonies which produce small pits of liquefaction; hence it has been called the "*Bacillus lacunatus*." In cultures it is distinctly pleomorphous, and involution forms also occur. It is non-pathogenic to the lower animals.

The *Diplobacillus liquefaciens* of Petit is practically identical in microscopic appearances with the bacillus of Morax, and is found in cases of conjunctivitis in which there is often a primary involvement of the cornea. It differs from the bacillus of Morax

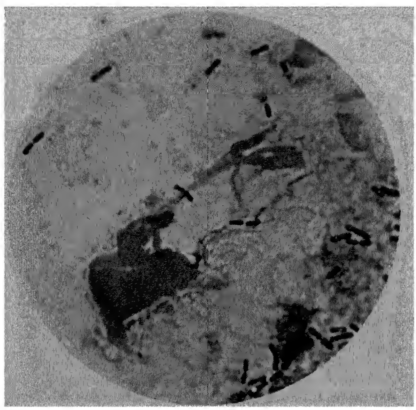


FIG. 37—Film preparation of conjunctival secretion, showing the Morax diplo-bacillus of conjunctivitis. ×1000.

in being able to grow on ordinary nutrient media and at 20° C. It liquefies gelatin at room temperature.

Bacillus xerosis, which is a small diphtheroid organism (Fig. 402), has been found in xerosis of the conjunctiva, in follicular conjunctivitis, and in other conditions; it occurs frequently also in the normal conjunctiva. It is doubtful whether it has any pathogenic action.

Acute conjunctivitis is produced by the pneumococcus, epidemics of the disease being sometimes due to this organism, and also by streptococci and staphylococci; *Staph. albus* is, however, found in the conjunctival sac, often in large numbers, as a normal commensal. True diphtheria of the conjunctiva caused by *B. diphtheriæ* also occurs, while in gonorrhœal conjunctivitis, often of an acute purulent type, the gonococcus is present (p. 370).

Bacillus coli Infections.—This group of organisms, which occur naturally as commensals in the bowel, are frequently associated with inflammatory and suppurative lesions in various parts of the body. Their biological characters will be dealt with in detail in Chapter XV.

Under experimental conditions the virulence of these organisms varies greatly and can be increased by passage from animal to animal. Injection into the serous cavities of rabbits produces a fibrinous inflammation which becomes purulent if the animal lives sufficiently long. If, however, the virulence of the strain be of a high order, death takes place before suppuration is established, and there is a septicæmic condition, the organisms occurring in large numbers in the blood. Intravenous injection of a few drops of a virulent broth culture usually produces a rapid septicæmia with scattered hæmorrhages in various organs.

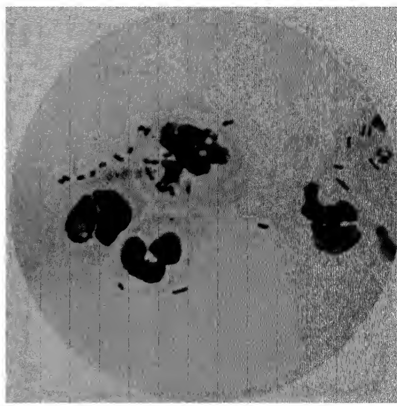


FIG. 38.—Film from urinary sediment showing *B. coli*. ×1000.

Bacillus coli is found in a great many inflammatory and suppurative conditions in connection with the alimentary tract—for example, in suppuration in the peritoneum, or in the extra-peritoneal tissue with or without perforation of the bowel, in the peritonitis following strangulation of the bowel, in appendicitis and the lesions following it, in suppuration in and around the bile ducts, etc. It may also occur in lesions in other parts of the body—endocarditis, pleurisy, etc., which in some cases are associated with lesions of the intestine, though in others such cannot be found. It is also frequently present in inflammation of the urinary passages, cystitis, pyelitis, abscesses in the kidneys, etc., these lesions being in fact most frequently caused by this group of organisms (Fig. 38).

We may remark that it has been repeatedly proved that the *Bacillus coli* cultivated from various lesions is more virulent than the ordinary intestinal strains, its virulence having been heightened by growth in the tissues.

Micrococcus tetragenus (*Gaffkia tetragena*, Trevisan).—This organism, first described by Gaffky, is characterised by the fact that it divides in two planes at right angles to one another and is thus generally found in the tissues in groups of four, or tetrads (Fig. 39), which are often seen to be surrounded by a capsule. The cocci measure about 0·6 to 0·8 μ in diameter. They stain readily with all the ordinary stains, and also retain the stain in Gram's method.

It grows readily in all the media at the room temperature. In a stab culture in gelatin a fairly thick whitish line forms along the track of the wire, while on the surface there is a thick rounded disc of whitish colour. The gelatin is not liquefied. On the surface of agar and of potato the growth is an abundant moist layer of the same colour. The growth on all the media may have a viscid character.

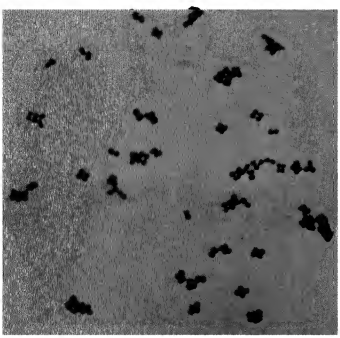


FIG. 39—*Micrococcus tetragenus*; young culture on agar, showing tetrads. Stained with weak carbol-fuchsin. ×1000.

Experimental inoculation.—White mice are exceedingly susceptible to this organism when recently isolated. Subcutaneous injection is followed by a general septicæmia, the organism being found in large numbers in the blood throughout the body. Guinea-pigs are less susceptible; sometimes only a local abscess with a good deal of necrotic change results; sometimes there is also septicæmia.

The *Micrococcus tetragenus* is often found in suppurations in the region of the mouth or in the neck, *e.g.* dental abscess, and also occurs in various lesions of the respiratory tract, in phthisical cavities, abscesses in the lungs, etc. Sometimes it is present alone, and probably has a pyogenic action in the human subject under certain conditions. In most cases it is associated with other organisms. Cases of general infection along with pneumonic symptoms have been recorded, the organism having been isolated from the blood; recovery was the rule. Cases of pyæmia have also been described in which this organism was found in a state of purity in the pus in various situations.

It should be noted that *Micrococcus tetragenus* may occur as a commensal on the mucous membrane of the upper respiratory passages. It has also been found on the skin.

Bacillus pyocyaneus (*Pseudomonas æruginosa*, Migula).—This organism occurs in the form of minute rods 1·5 to 3 μ in length and about 0·5 μ in thickness (Fig. 40). Occasionally two or three are found attached end to end. It is actively motile, possessing one to three terminal flagella, and does not form spores. It stains readily with the ordinary basic stains, but is decolorised by Gram's method.

Cultivation—It grows readily under aerobic conditions on all

the ordinary media at room temperature, the cultures being distinguished by the formation of a greenish pigment. The optimum temperature is about 37° C. On agar the growth forms an abundant slimy greyish layer which afterwards becomes greenish, and a bright green colour diffuses through the whole substance of the medium. In stab cultures in gelatin a greyish line appears in twenty-four hours, and at its upper part a small cup of liquefaction forms within forty-eight hours. At this time a slightly greenish tint is seen in the superficial part of the gelatin. The liquefaction extends rapidly, the fluid portion being turbid and showing masses of growth at its lower part. The green colour becomes more marked, and diffuses through the gelatin. Ultimately liquefaction reaches the wall of the tube. In plate cultures the colonies appear as minute whitish points, those on the surface being the larger. Under a low power of the microscope they have a brownish yellow colour and show a nodulated surface, the superficial colonies being thinner and larger. Liquefaction soon occurs, the colonies on the surface forming shallow cups with small irregular masses of growth at the bottom, the deep colonies small spheres of liquefaction. Around the colonies a greenish tint appears. On potato the growth is an abundant reddish-brown layer resembling that of the glanders bacillus (*q.v.*). Under anaerobic conditions the pigment becomes reduced to a colourless "leuco" compound, but the colour returns on oxygenation.

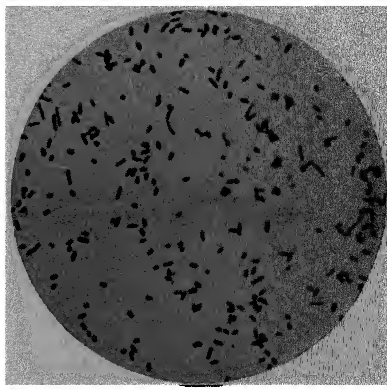


FIG. 40.—*Bacillus pyocyaneus*; young culture on agar. Stained with weak carbol-fuchsin. ×1000.

From the cultures there can be extracted by chloroform a blue-green pigment, pyocyanin, which crystallises in the form of long, delicate bluish-green needles. On the addition of a weak acid its colour changes to red. A yellowish-green fluorescent pigment, fluorescein, is also produced, differing from pyocyanin in its solubility in water and insolubility in chloroform. When continuously cultivated in the laboratory the organism may lose the power of forming pyocyanin. A red pigment, pyorubin, has also been described.

Under cultural conditions *B. pyocyaneus* exerts an antagonistic effect on certain other bacteria *e.g. B. anthracis*, and this has been attributed to a lytic ferment-like agent, pyocyanase, which can be demonstrated in filtrates of broth cultures.

Experimental Inoculation.—*Bacillus pyocyaneus* has distinct pathogenic action in certain animals. Subcutaneous injection of small doses in rabbits may produce a local suppuration, but if the dose be large, spreading hæmorrhagic œdema results, which may

be attended by septicæmia. Intravenous injection may produce, according to the dose, rapid septicæmia with nephritis, or sometimes a more chronic condition of wasting attended by albuminuria

Lesions in human subject—*Bacillus pyocyaneus* (the organism of "green" or "blue" pus) is rarely found alone, though it is not infrequent along with other organisms. We have met with it several times in cases of multiple abscesses, in association with the *Staphylococcus aureus*. It is present along with other organisms in a proportion of suppurating wounds. Cases of disease in children have been described in which *Bacillus pyocyaneus* has been found throughout the body; in these cases the chief symptoms have been fever, gastro-intestinal irritation, pustular or petechial eruptions in the skin, and general marasmus. It sometimes occurs in cystitis and pyelitis, and in otitis

This organism is found naturally in decomposing organic matter, in sewage, water, and soil.

Bacillus proteus.—The term *Proteus* has been applied to a group of bacteria, of which several varieties have been described, e g. *vulgaris, mirabilis, zenkeri, capsulatus*; the *Urobacillus septicus* is also a variety. They are characterised by their pleomorphism, hence the name, and by their rapid liquefaction of gelatin. *B. proteus* has the following characters: it is a small bacillus, 1 to 3 μ by 0·5 μ, straight or slightly curved, but coccoid and filamentous forms also occur, and a marked tendency to involution forms is to be noted. It is actively motile, and possesses numerous lateral flagella; it does not form spores. It stains readily with basic dyes and is Gram-negative. It grows readily on all the ordinary media at room temperature, but best between 25° and 37° C. On an agar slope the organism forms a moist layer, which extends over the whole surface of the medium; in this way the bacillus can readily be separated from other organisms present along with it, but in mixed culture other organisms are difficult to separate from *B. proteus* owing to its spreading growth. This type of organism is therefore spoken of as a "spreader"; the same spreading growth is noted in plate cultures but is not invariably present In a gelatin stab culture liquefaction appears within twenty-four hours; it develops rapidly in the form of a crater, and ultimately the whole medium undergoes stratiform liquefaction and presents a turbid appearance In gelatin plates the characters are somewhat peculiar, especially when 5 per cent. gelatin is used. The colonies are at first small spheres with granular centres and peripheral radiations extending into the medium; liquefaction soon follows, and from the superficial colonies offshoots extend over the medium in tendril-like fashion, these being composed of bacilli in chains placed side by side. Groups of bacilli may also become separate, move over the surface of the medium, and form growths at a distance—the so-called "swarm-colonies." On potato it forms a slimy film with a greyish-brown colour. It coagulates milk (without acid reaction) and later digests the casein. The organism is actively proteolytic and forms sulphuretted hydrogen, reduces nitrates to nitrites and ultimately to ammonia, and splits urea. Coagulated serum is liquefied by some strains. It forms acid and gas from glucose and does not ferment lactose or mannitol, but its action on saccharose and maltose varies. Indole is produced by certain strains. The addition to agar medium of

1 : 2000 copper sulphate prevents *B. proteus* from producing the spreading type of growth, so that isolated colonies may be obtained (Allison and Ayling). Rough and smooth types of colony may be distinguished. Non-motile variants (O type) may occur and the growths of these do not "spread" like those of the typical form. A form of *B. proteus* has been isolated from the urine in typhus fever which is of special type ("X 19"); maltose is fermented and indole formed by it. It differs from other strains in its fermentation of salicin. The fact that this organism is agglutinated by the serum in typhus fever (Weil-Felix reaction) is of great value in diagnosis (p. 709). The agglutinin which yields the Weil-Felix reaction is of the O type. *B. proteus* X 19 differs serologically from other strains. A further variety named "Kingsbury" is agglutinated by the serum from cases of a typhus-like illness in Malaya and this reaction is also applied for diagnostic purposes. This question will be discussed further in the chapter dealing with Typhus Fever.

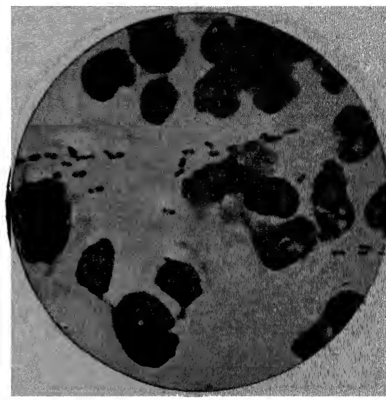


Fig. 41.—Film preparation of pus from soft chancre, showing Ducrey's bacillus, chiefly arranged in pairs. Stained with carbol-fuchsin and slightly decolorised. ×1500[1].

Experimental Inoculation—Strains of this organism isolated from septic lesions when injected subcutaneously in laboratory animals produce either a localised inflammatory lesion with pus formation or a spreading inflammatory œdema and septicæmia with fatal result.

Lesions in human subject.—Although some cases of pure infection by this organism have been described, *e.g.* pleurisy, meningitis from middle-ear disease, etc., the bacilli generally occur along with other organisms in septic inflammations, such as cystitis and pyelitis, endometritis, peritonitis, etc. *B. proteus* is common also in gun-shot and other contaminated wounds Cases of gastro-enteritis, including food-poisoning, have been attributed to *B. proteus* by certain observers; and the organism is a frequent concomitant of *B. dysenteriæ* infection, occurring often in large numbers in the stools in late stages of the disease.

It occurs naturally in putrefying organic material and is found frequently in fæces.

Organisms which are probably related to *B. proteus* have been described in ozæna, *e g. Coccobacillus fœtidus ozænæ* of Perez. They appear to be responsible for the fœtor and secondary effects in this condition.

Ducrey's Bacillus of Soft Sore (Chancroid).—The bacillus of soft sore (*Hæmophilus ducreyi*) was first described by Ducrey

[1] We are indebted to Dr. Davis for the use of Figs. 41 and 42.

in 1889, who found it in the purulent discharge from the ulcerated surface; and later, in 1892, Unna described its appearance and distribution as seen in sections through the sores.

Microscopical Characters.—The organism occurs in the form of minute oval rods measuring about 1·5 μ in length, and 0·5 μ in thickness (Fig. 41). It is found mixed with other organisms in the purulent discharge from the surface, and is chiefly arranged in small groups or in short chains. When studied in sections through the ulcer, it is found in the superficial part of the floor, but more deeply situated than other organisms, and may be present in a state of purity in the leucocytic infiltration. In this position it is usually arranged in chains, which may be of considerable length, and which are often seen lying in parallel rows between the cells. The bacilli chiefly occur in the free condition.

There is no doubt that in many cases the organism is present in the buboes in a state of purity; it has been found there by microscopic examination, and cultures have also been obtained from this source. Sometimes the ordinary pyogenic organisms become superadded. A method of demonstrating the presence of the organisms where they are scanty consists in injecting pus or secretion from the patient intracutaneously into himself; after two or three days Ducrey's bacillus can often be found readily in the contents of the resulting lesion (Ravaut, Rabeau and Hesse).

The bacillus in film preparations can readily be stained by most of the ordinary dye solutions, though Löffler's methylene-blue solution is preferable, as it does not over-stain. In sections, however, great care must be taken in the process of dehydration, and the aniline-xylol method (*vide* p. 112) should be used for this purpose, as alcohol decolorises the organism very readily. Ducrey's bacillus reacts negatively in Gram's method.

Cultivation.—For a long period of time attempts to obtain cultures were unsuccessful. Benzançon, Griffon and Le Sourd obtained pure cultures in four cases, the medium used being a mixture of rabbit's blood and agar, in the proportion of one part of the former to 2 of the latter. The blood was added to the agar in the melted condition at 45° C., and the tubes were then sloped. Davis confirmed these results, and found that another good medium is freshly drawn human blood distributed in small tubes; this method is specially suitable, as the blood inhibits the growth of various extraneous organisms. Initial cultures from sores can also be obtained readily in tubes containing 1 or 2 c.c. of sterile rabbit's blood which has been allowed to coagulate and has then been heated at 55° C. for five minutes. The primary growth obtained in this way is subcultured on blood agar. Cultures may be made by this method from the buboes, the inoculum being obtained by puncturing the enlarged gland with a syringe. On solid medium the growth appears in the form of very small round globules, which after forty-eight hours do not exceed 1 mm. in diameter; the colonies do not become confluent. The organism shows hæmolytic properties. Microscopic examination of these colonies, which are dissociated with some difficulty, shows appearances similar to those observed when the organism is in the tissues (Fig. 42), but occasionally long undivided filaments are observed. Within a comparatively short period cultures undergo marked degenerative changes, and great irregularities of form and shape

are to be found. It would appear that a comparatively large amount of blood is necessary for the primary growth of this organism. Teague and Deibert have shown that the growth-promoting principle is present in erythrocyte extracts. After the organism is accustomed to artificial medium, serum media or even ordinary media may support its growth. Inoculation of the ordinary laboratory animals is not attended by any result, but it has been found that some monkeys are susceptible, small ulcerations being produced by superficial inoculation, and in all these the organism can be demonstrated. Tomasczewski cultivated the organism for several generations, and reproduced the disease by inoculation of the human subject. The causal relationship of this bacillus must therefore be considered as completely established, and the conditions under which it grows show it to be a strict parasite under natural conditions—a fact which is in conformity with the known facts as to the transmission of the disease. A cutaneous allergic reaction can be elicited in infected persons by intradermal injection of the killed organisms. This reaction shows a high degree of specificity (Reenstierna).

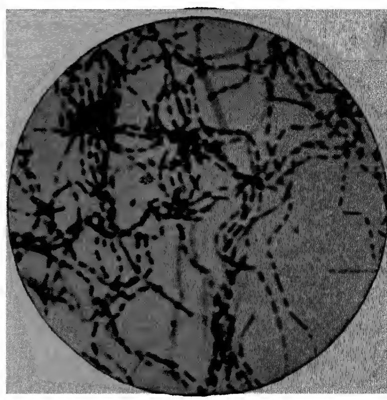


FIG. 42.—Ducrey's bacillus from a twenty-four-hours' culture in blood-broth. ×1500.[1]

Vaccine Treatment of Infections by the Pyogenic Organisms.—From his study of the part played by phagocytosis in the resistance of the body to the pyogenic bacteria, Wright was led to advocate the treatment of such infections during their course by active immunisation by means of dead cultures of the infecting agent (for methods of preparation, see p. 158). The treatment is specially applicable when the infection is practically local, as in acne pustules, in boils, etc., but has also been applied in more acute conditions. (For the theoretical questions raised, see Immunity.) For an isolated furuncle, Wright recommended a dose of 50 to 100 millions staphylococci to be followed three or four days later by the injection of 250 to 300 millions, and for an incipient streptococcal lymphangitis a dose of 500,000 to 2,000,000 streptococci. In chronic staphylococcal infections the number of bacteria used for an injection is from 250,000,000 to 500,000,000, but a smaller number may give a good result, and the general principle to be adopted is to use the smallest dose necessary for a therapeutic effect. If it is not practicable to use the strain derived from the lesion for the preparation of an " autogenous " vaccine, then laboratory cultures

[1] See Footnote, page 321.

or the stock vaccines which are on the market may be used; in such cases it is well to use a "polyvalent" vaccine made from a mixture of strains. The treatment of various staphylococcus infections, such as pustular acne, boils, and chronic abscesses, by vaccines, has been carried out very extensively, in many cases with good result, and a similar statement is true of some streptococcal infections. Vaccine therapy has also been used in other inflammatory and suppurative conditions, for example, infections of the genito-urinary tract with *B. coli*, where an autogenous vaccine with initial doses of 10,000,000 to 50,000,000 may be employed, chronic respiratory catarrh, etc. The case of the latter can usually only be met by mixed vaccines on account of the presence of different species of bacteria, several of which may be potentially pathogenic; in these circumstances the use of a mixed vaccine is purely empirical.

The treatment has also been applied in acute infections, *e.g.* with the pyogenic cocci, *B. coli*, etc., very small doses—*e.g.* from 200,000 to 5,000,000—being given, but the method has not been attended by any degree of success.

Methods of Examination in Inflammatory and Suppurative Conditions.—These are usually of a comparatively simple nature, and include (1) microscopic examination, (2) the making of cultures.

(1) The pus or other fluids should be examined microscopically, first of all by means of film preparations in order to determine the characters of the organisms present. The films should be stained (*a*) by one of the ordinary solutions, such as dilute carbol-fuchsin or a watery solution of methylene-blue; and (*b*) by Gram's method (p. 116). The use of the latter is of course of great importance as an aid in the recognition of the pyogenic organisms.

(2) The cultivation and separation of the organisms from the lesions are best attained by the method of successive strokes on agar plates or on agar slopes, the former being preferable (p. 88). In the case of an organism requiring a special medium, this of course is to be used. In the routine examination of streptococcal infections blood agar may be used with advantage to distinguish the hæmolytic and non-hæmolytic types. Animal inoculation experiments may be carried out as occasion arises.

In cases of suspected blood infection the examination of the blood is to be carried out by the methods already described (p. 165).

Reference is made on p. 165 *et seq.* to the methods of obtaining suitable specimens for bacteriological examination.

CHAPTER VIII

BACTERIA ASSOCIATED WITH INFLAMMATORY AND SUPPURATIVE CONDITIONS (*continued*): THE PNEUMOCOCCUS AND PNEUMOBACILLUS; THE MENINGOCOCCUS AND GONOCOCCUS.

The Pneumococcus and Acute Pneumonia

Introductory.—Inflammatory changes in the lungs are the results of infection in different ways, and lead to a variety of structural changes. Thus different forms—lobar pneumonia, broncho-pneumonia, hypostatic pneumonia, and embolic pneumonia—are recognised. Acute lobar pneumonia, however, stands out in certain respects from the other forms and presents characters of special interest. Its striking clinical features and the course of the temperature, especially its critical fall, have long been recognised as resembling those of an acute specific infection. Further, its occurrence in epidemics supports the view that it is of the nature of a specific infection. Our knowledge of its etiology has been gradually evolved, rather than established by one discovery as has been the case with other infections; and years elapsed after the discovery of the pneumococcus before it gained general acceptance as the causal organism. This was due to several circumstances, but chiefly to the fact that the organism was found both in other lesions and in normal throats, and also to the difficulty experienced in producing a typical lobar pneumonia in animals.

Historical.—The first important work on the etiology of pneumonia was that of Friedländer (1882–83), who observed in the lungs capsulated cocci, which he isolated and showed to possess pathogenic properties. The situation was complicated by the subsequent observation that the injection into animals of the sputum of healthy individuals frequently originated a septicæmic condition with the presence of capsulated cocci in the blood. The significance of the occurrence of this "sputum septicæmia" could not at that period be properly realised, as it was not recognised that an organism could produce different results in different animals, and therefore it was thought that the organisms described by Friedländer were not specifically related to pneumonia. Somewhat later, A. Fraenkel

described diplococci in pneumonia which differed culturally from those of Friedländer. The work of Weichselbaum in 1886 elucidated the subject further. This observer, investigating 129 cases of various types of pneumonia, isolated, first and most frequently, an organism which he called the *Diplococcus pneumoniæ* (with a variant named by him the *Streptococcus pneumoniæ*), and which corresponded with Fraenkel's organism; second, an organism which he described as the *Bacillus pneumoniæ*, occurring less frequently and corresponding with that originally noted by Friedländer.

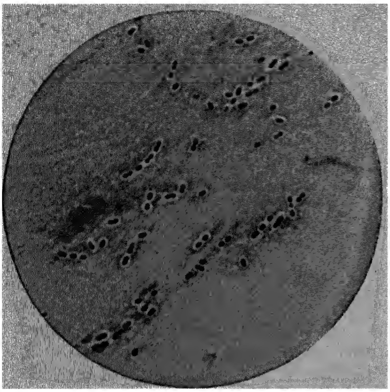


FIG. 43.—Film preparation of pneumonic sputum, showing numerous pneumococci (Fraenkel's) with unstained capsules; some are arranged in short chains. See also Plate I., Fig. 2.
Stained with carbol-fuchsin. ×1000.

The general result of all observations on pneumonia has been to establish that the organism described by Fraenkel and now known as the pneumococcus is that of most frequent occurrence; it is the sole organism present in about 95 per cent. of cases of lobar pneumonia.

Pneumococcus (*Diplococcus pneumoniæ*): *Microscopic Characters.* — As seen in pneumonic sputum or exudate, the pneumococcus occurs in the form of a small oval coccus, about 1 μ in longest diameter, arranged generally in pairs (diplococci), but also in chains of four to ten (Fig. 43). The free ends are often pointed like a lancet; hence the name "*Diplococcus lanceolatus*" has also been applied to it. These cocci, in their typical form, have round them a capsule, which in films stained by ordinary

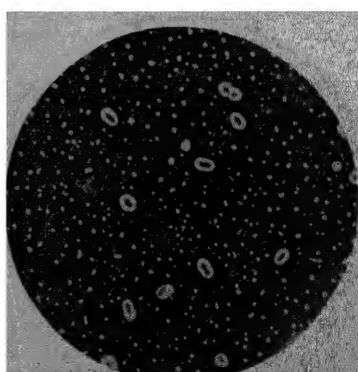


FIG. 44.—Pneumococcus; film from lung exudate in lobar pneumonia. Eosin capsule method. ×1000.

methods usually appears as an unstained halo, but is sometimes stained more deeply than the ground of the preparation. This difference in staining depends, in part at least, on the amount of decolorisation to which the preparation has been subjected. The capsule is rather broader than the body of the coccus, and has a sharply defined external margin (Fig. 44); it may be stained by the special methods already described (Fig. 45). In sputum preparations the capsule of the pneumococcus may not be recognisable, and the same is sometimes true of preparations of pneumococcal exudates in the lung or in other parts of the body. A variety of pneumococcus is recognised ("*P. mucosus*") whose capsule is particularly well developed both in the tissues and in culture; the cocci in this form tend to be more spheroidal than the typical varieties. The pneumococcus takes up the basic aniline stains with great readiness and is Gram-positive. In any lesion many degenerate individuals may occur, and these have often become Gram-negative.

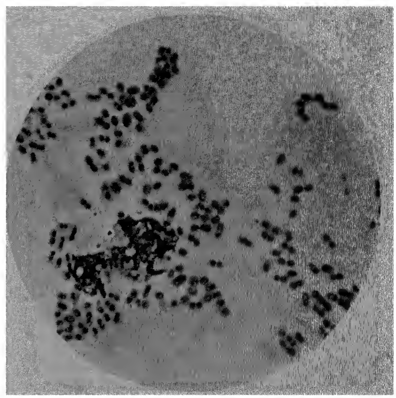


FIG. 45.—Pneumococcus in serous exudation at site of inoculation in a rabbit, showing capsules.
Stained by Rd. Muir's method. ×1000.

Cultivation.—To obtain a pure culture it is best to inject sputum beneath the skin of a rabbit or a mouse. In about twenty-four to forty-eight hours the animal will die, with numerous capsulated pneumococci throughout its blood. From the heart-blood pure cultures can easily be obtained. Pure cultures can also be got *post mortem* from the lungs of pneumonic patients by inoculating blood-agar with a scraping taken from the area of acute congestion or commencing red hepatisation, and incubating at 37° C. Cultures made from pneumonic sputum usually yield a mixed growth with varying numbers of pneumococci present.

The pneumococcus is an aerobe and facultative anaerobe, and its optimum temperature is about 37° C.; growth does not occur as a rule below 25° C. It grows on ordinary media,

but best on serum- or blood-agar, or on boiled-blood-agar. The colonies are small (1 mm. diameter) and very transparent, but under a low power of the microscope appear to have a compact finely granular centre and a pale transparent periphery; after forty-eight hours they increase slightly in size and present a depressed centre and raised border. The appearances are similar to those of a culture of *Streptococcus*, but the growth is slightly less vigorous and more delicate in appearance. "*Pneumococcus mucosus*" (*vide supra*) produces relatively large colonies of mucoid consistence (p. 331).

Growing on *blood-agar* the pneumococcus produces a greenish coloration of the medium, attributed to methæmoglobin formation. There is also partial hæmolysis. Under anaerobic conditions complete hæmolysis may occur without green coloration. The hæmolysin of the pneumococcus has been studied by Cowan who found that it appears in young cultures after the logarithmic phase, but is inactivated by oxidation; it is filterable, labile at 55° C. and antigenic. On heated blood the growth is greenish-yellow, like that of *Strept. viridans*. McLeod and Gordon have found that the pneumococcus produces hydrogen peroxide, whilst there is only slight formation of catalase; the peroxide formed, not being destroyed by catalase, is a factor in inhibiting growth. The organism is active in producing either oxidation or reduction, according to the tension of free oxygen present (Neill and Avery). The same processes occur in filtrates and lysates of cultures. Thus hæmoglobin is converted to methæmoglobin in the presence of oxygen, but at a low oxygen tension a reversal of this process takes place. In *broth*, growth forms at first a uniform turbidity, but tends to settle at the bottom of the vessel as a dust-like deposit.

For full growth of the pneumococcus, according to H. D. Wright, a small amount of fermentable carbohydrate, *e.g.* 0·2 per cent. glucose, is necessary in the culture medium; he states also that 1 per cent. peptone affords a suitable supply of thermostable nitrogenous substances, while yeast extract, blood, meat extract, and serum contain the thermolabile constituents which are essential for growth. Heating of organic fluids used for culture media, *e.g.* peptone, serum, and yeast extract renders them inhibitory to growth and one of the difficulties encountered in growing this organism is its great susceptibility to such inhibitory influences. A suitable broth medium is that described on p. 56.

Cultures may be maintained for long periods if fresh subcultures are made every two or three days, but they tend ultimately to die out. They sometimes rapidly lose their viru-

lence, so that four or five days after isolation from an animal's body their pathogenic action disappears ; but this is not always the case, especially if blood or serum media be used for maintaining subcultures, or if subcultures are made at very short intervals. The pneumococcus is comparatively sensitive to external agencies. The thermal death-point is about 52° C. In culture it soon dies when dried. When present in dry sputum or blood, however, it is more resistant and may retain its vitality for a considerable time. A convenient method to pre-

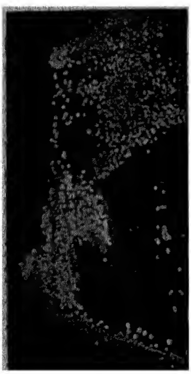


Fig. 46.—Stroke culture of pneumococcus on blood-agar. Twenty-four hours' growth at 37° C. Natural size.

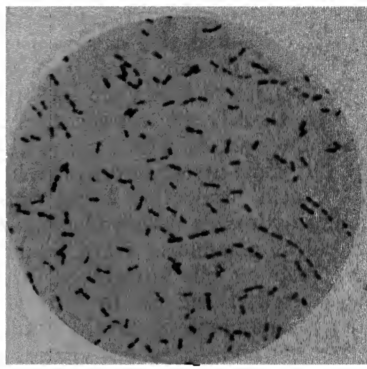


Fig. 47.—Pneumococcus from a pure culture on blood-agar of twenty-four hours' growth, some in pairs, some in short chains.
Stained with weak carbol-fuchsin. ×1000.

serve the organism is to dry thoroughly the spleen of a mouse dead of pneumococcal septicæmia. In the dried tissue the organism not only remains alive, but may retain its virulence. In ordinary artificial media pneumococci usually appear as diplococci without a capsule, but in preparations made from the surface of agar or from broth, shorter or longer chains may be observed (Fig. 47). After a few days' growth they lose their regular shape and size, and involution forms appear. Usually the pneumococcus does not grow below 22° C., but strains in which the virulence has disappeared often grow well at 20° C. In ordinary media, as just stated, the organism does not usually appear to develop a capsule, but as Hiss showed, the absence of a capsule is often only apparent, and if in making cover-glass preparations from such media some serum be used as the

diluent, and the films be stained by his copper-sulphate method (p. 122), a capsule can be demonstrated. Capsulation frequently appears in fluid serum media.

As in other bacterial species, variation associated with changes in colony characters, etc., is noted in cultures. Thus a culture with the typical " smooth " type of colony may undergo alteration to a " rough " form, the colonies becoming thicker, heaped up, more opaque, more coherent, and with a granular surface. This change is associated with variation in other features which will be dealt with later.

The pneumococcus ferments saccharose, raffinose, and lactose ; a similar fermentative action on inulin is important, as most streptococci do not ferment this carbohydrate. Apparently some samples of inulin are less suitable for this reaction than others. Usually the test is carried out with Hiss's inulin serum water medium, in which coagulation of the serum results (p. 79), but some investigators have had more success with inulin broth, acid production being estimated by titration against sodium hydroxide with a phenol-phthalein indicator.

Virulent pneumococci are soluble in bile. To demonstrate this, fresh ox bile autoclaved for twenty minutes at 120° C. and filtered, is added to a well-grown fluid culture (which must be one in simple broth) in the proportion of about a fifth of the culture. Two per cent. sodium taurocholate may be similarly used, but such solutions may be somewhat variable in their effect. It is supposed that bile acts by hastening the natural autolysis of the organisms. The reaction should be carried out at a pH 7·6. It should be noted that " rough " variants of the pneumococcus may sometimes be bile-insoluble.

Mair recommends as an improvement in the test the use of a 10 per cent. solution of sodium desoxycholate, alkaline to phenolphthalein. Of the solution 0·1 c.c. is added to 5 c.c. of a serum broth culture of the organism ; clearing of the mixture occurs in ten to fifteen minutes. If the culture is acid, precipitation of the bile acids may occur, but this disppears on the addition of a drop or two of normal caustic soda.

The facts that in cultures the pneumococcus often grows in chains, and that occasionally streptococci are found to develop capsules, have raised the question of the relationship of this organism to the streptococci. In identifying the pneumococcus, biological as well as morphological characters must be studied, and bile-solubility and fermentation of inulin are important characters. It may be stated, however, as bearing on the close relationships of the pneumococcus and streptococci, that Rosenow believes he has succeeded in transforming strepto-

cocci into capsulated organisms having all these biological features of the pneumococcus. Morgenroth and his co-workers, by treating pneumococci in various ways by optoquine, claim to have succeeded in effecting transformation into *Streptococcus viridans* and less frequently into a hæmolytic streptococcus ; a reversion was also met with, but only rarely. These results are of importance, but further investigation of the subject is required.

It is noteworthy that coccal types are met with which show characters intermediate between the true streptococci and the pneumococcus, *e g.* Aronson's streptococcus (*vide* p. 292). Atten-

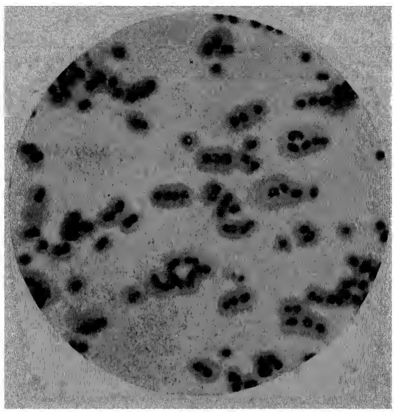


FIG. 48.—*Pneumococcus mucosus* (Type III.) from peritoneal fluid of mouse. Capsule stained. ×1000.

tion may also be drawn here to a group of cocci originally described by Schottmuller, isolated from various diseased conditions in man (pneumonia, otitis, meningitis, suppurations), which besides possessing voluminous capsules have these surrounded by a viscous material which gives a slimy consistence to cultures and also to pathological exudates. These are related to the pneumococcus on the one hand and to the streptococci on the other. The work of the Rockefeller Institute investigators (*vide infra*) suggests that these organisms ought to be classified into two groups. (1) The *Pneumococcus mucosus* (Fig. 48) : this organism tends to be less pointed than the typical pneumococcus, and its colonies are larger and mucoid ; its action on blood agar is like that of the typical pneumococcus (*vide supra*), it is soluble in bile, ferments inulin, and is very pathogenic to white mice and rabbits. Antisera produced by strains of this coccus, while showing agglutination towards members of their own group, do not agglutinate streptococci and usually also not other pneumococci. (2) The *Streptococcus mucosus* (Fig. 49) : this organism is generally round, occurs in chains,

and the colonies are less transparent than those of the pneumococcus; it is usually non-hæmolytic, is not soluble in bile, does not ferment inulin, and is less pathogenic to mice than the last. Thus while the *Pneumococcus mucosus* is practically a true pneumococcus, the *Streptococcus mucosus* forms a connecting link with the true streptococci. It has also been found that the mucous capsules may disappear on animal passage and may thus represent a reaction of the organism developed under special conditions in the original host (Browning and Gulbransen).

Experimental Inoculation.—The pneumococcus is pathogenic to various animals, though the effects vary somewhat with the virulence of the strain used. The susceptibility of differ-

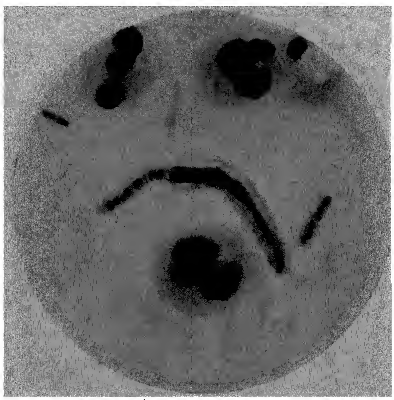


FIG. 49.—*Streptococcus mucosus* in pus. Stained with Löffler's methylene-blue. ×1000.

ent species, as Gamaleia showed, varies to a considerable extent. The rabbit, and especially the mouse, are very susceptible; the guinea-pig, the rat, the dog, and the sheep occupy an intermediate position; the pigeon is immune. In the more susceptible animals the general type of the disease produced is not pneumonia, but a *septicæmia*. Thus, if a rabbit or a mouse be injected subcutaneously with pneumonic sputum, death occurs in from twenty-four to forty-eight hours. There is some fibrinous infiltration at the point of inoculation, the spleen is often enlarged and firm, and the blood contains capsulated pneumococci in large numbers (Fig. 50).

When relatively avirulent cultures are used, local inflammatory changes are set up in susceptible animals instead of septicæmia, and corresponding results are said to follow when virulent cultures

are injected into such animals partially immunised. So also in the more resistant animals, such as the sheep and the dog, the lesions produced are of inflammatory type, and when the injections are made into the lungs pneumonia may be set up Lamar and Meltzer by intra-bronchial insufflation of cultures of pneumococci in the dog, succeeded in producing typical lobar pneumonia; although, in the cases where recovery followed, the general course of the infection did not fully correspond with that in the human subject. Comparatively large amounts of culture were used. The most conclusive experiments on the subject, however, are those of Blake and Cecil who, by intra-

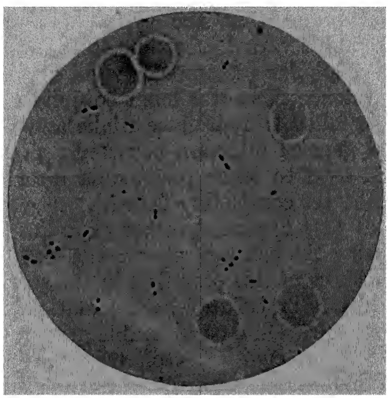


FIG 5).—Capsulated pneumococci in blood taken from the heart of a rabbit, dead after inoculation with pneumonic sputum.
Dried film, fixed with corrosive sublimate. Stained with carbol-fuchsin and partly decolorised. ×1000.

tracheal injection in monkeys of virulent pneumococci in minute doses, were able regularly to set up a lobar pneumonia which ran a course like that of the human disease, recovery taking place by crisis. On the other hand, the introduction in larger amounts of similar pneumococci into the throat or nose of these animals was not followed by pneumonia, even though the organisms persisted for a considerable time. This is of importance in connection with the natural occurrence of pneumonia, as it points to some additional factor being necessary for its origin, especially in view of the fact that monkeys are apparently more susceptible than the human subject. Blake and Cecil met with spontaneous pneumonia in monkeys kept in confinement, and the disease, which had the same features as

that experimentally produced, readily passed from animal to animal. They came to the conclusion that when virulent pneumococci are introduced into the trachea they probably penetrate the wall of a large bronchus near the root of the lung, and thence spread by the interstitial tissue outwards from the hilum; a general inflammation of the substance of the lung is thus produced. They were unable to set up lobar pneumonia in monkeys by subcutaneous or by intravenous injection; on the other hand, secondary invasion of the blood stream by the pneumococci occurred in the experimentally produced lobar pneumonia, the number of organisms increasing in cases which terminated fatally. Gaskell, using adjusted doses by the intratracheal method found that the types of pneumonia thus produced in the rabbit varied according to the virulence; with a certain strain a lobular pneumonia might result, while with one of higher virulence there was lobar consolidation. McLeod and Gordon have failed to reproduce Blake and Cecil's results, although a fatal general infection occurred; accordingly, the conditions which lead to lobar pneumonia after intratracheal inoculation are not yet completely defined.

A fact which at first appeared rather to militate against the pneumococcus being the cause of pneumonia was the discovery by Pasteur and others of this organism in the saliva of healthy men. It can certainly be isolated by inoculation of susceptible animals, from the mouths of a considerable proportion of normal men, from their nasal cavities, etc., being probably in any particular individual more numerous at some times than at others (especially, it is stated, during the winter months). It must be noted, however, that according to the Rockefeller Institute investigations (*vide infra*) the pneumococcus occurring in the healthy naso-pharynx is usually of Group IV., *i.e.* belongs to those least pathogenic to man. The more pathogenic types are found almost exclusively in the mouths of convalescents and of contacts and in rooms where pneumonia cases have been nursed. While these types usually disappear rapidly from convalescents and contacts they may persist long enough to justify the view that certain persons may act as carriers of the disease. The exact conditions leading to infection are imperfectly understood, but we must recognise the importance of predisposing causes in the etiology of the disease, as in the case of the diseases caused by pyogenic staphylococci, streptococci, the *Bacillus coli*, etc. By such causes the vitality and power of resistance of the lung may be diminished, and then the pneumococcus gains an entrance. We can

therefore understand how such factors as cold, alcoholic excess, abnormal conditions of the respiratory tract—a slight bronchitis, etc.—can play an important part in the causation of pneumonia.

The experiments of Webster and Clow are of special interest in relation to the pathogenicity of the pneumococcus and the epidemiology of infection by this organism. They found that the pneumococcus freshly isolated from the human subject and instilled in small doses into the nose of mice brought about a characteristic infection which spread to healthy contacts, producing in them either the carrier state or a fatal infection. The difference in pathogenic effects among individual animals ranged from the refractory state or a local cervical lymphadenitis to fatal lobular or lobar pneumonia with or without pleurisy, empyema, pericarditis or septicæmia. Considerable differences were also noted among strains as regards their virulence on intranasal instillation. A further interesting feature of the pathogenicity of the pneumococcus has been elicited by Goodner who found that a dermal lesion could be produced in rabbits by the intradermal injection of the virulent organism and that this condition sometimes terminated by "crisis," though it usually led to fatal septicæmia.

It is of interest that extracts of pneumococcus cultures have been found to possess "thrombolytic" properties and when injected into animals produce a decrease of blood platelets with a resulting condition of purpura. It is supposed that the active substance is a derivative of autolysis (Mair; Julianelle and Reimann).

The Occurrence of the Pneumococcus in Pneumonia and other Conditions.—The pneumococcus occurs in every variety of the disease—in acute lobar pneumonia, in bronchopneumonia, in septic pneumonia. In a case of lobar pneumonia the pneumococci are found all through the affected area in the lung, especially in the exudation in the air cells. They also occur in the pleural exudate, and in the lymphatics of the lung. The greatest number are found in the parts where the inflammatory process is most recent. When the inflammation is resolving, some of the organisms often stain badly (*e.g.* tend to lose the Gram-positive reaction); such individuals are probably either dead or dying. Sometimes there occur in pneumonic consolidation areas of suppurative softening, which may spread diffusely. In such areas the pneumococci occur with or without ordinary pyogenic organisms, streptococci being the commonest concomitants. In other cases, especially when the condition is secondary to influenza, gangrene may supervene and lead to destruction of large portions of the lung. In these a great

variety of bacteria, both aerobes and anaerobes, are to be found. By direct extension to neighbouring parts, empyema, pericarditis, and lymphatic enlargements in the mediastinum and neck may take place; the organism may occur either alone or with pyogenic cocci.

In a considerable proportion of cases of lobar pneumonia, pneumococci can be cultivated from the blood. This represents a bacteriæmia, due to a secondary extension of organisms from the lung, and varies in degree in different cases. When the number is considerable the effects are liable to be severe; according to some observers a number of over fifteen cocci per c.c. is of grave import. It is interesting to note that in the lobar pneumonia experimentally produced by Blake and Cecil in monkeys, a progressive increase of pneumococci in the blood was accompanied by a progressive diminution in the number of leucocytes and led to a fatal result. The presence of pneumococci in the blood explains the occurrence of such inflammatory complications as meningitis, suppurations in connective tissue, joints, etc. (A primary meningitis, apart from pneumonia, may be produced by the pneumococcus, p. 361.) Ulcerative endocarditis may also develop, sometimes after the occurrence of a distinct crisis, and in this connection we may refer to the interesting observation made by Mair, namely, that in rabbits partially immunised by dead cultures intravenous injection of virulent pneumococci is followed by endocarditis in a large proportion of cases. The pneumococci obtained from the vegetations were found to have lost their virulence.

Other Types of Pneumonia and other Primary Pneumococcal Infections.—The so-called "simple" or non-suppurative type of broncho-pneumonia common in children shows certain points of difference from the lobar type. It represents a pneumonia starting as multiple foci, usually in both lungs, and is the result of spread of inflammatory change from the terminal bronchioles along the air passages to the alveoli, although extension through the walls of the bronchioles also takes place. There is an absence of the rapid general involvement of the interstitial tissue of the lung seen in lobar pneumonia, and an important point is that it is characterised by the absence of crisis. In broncho-pneumonia in children the pneumococcus is again an important causal agent and can be found in many cases. Other organisms of the pyogenic group may occur along with it or sometimes alone. *B. influenzæ* is also frequently found. In the adult the proportion of cases caused by organisms other than the pneumococcus is somewhat greater. In the broncho-pneumonia second-

ary to diphtheria the pneumococcus may be accompanied by the diphtheria bacillus and also by pyogenic cocci. In typhoid pneumonia the typhoid bacillus may be alone present or may be accompanied by the pneumococcus. In septic pneumonias the pyogenic cocci in many cases are the only organisms discoverable, but the pneumococcus also may be present. In influenza there is a general lowering of the resistance of the bronchial mucosa and a great variety of organisms, including *B. influenzæ*, pneumococcus, streptococci, staphylococci, and *M. catarrhalis*, etc., are enabled to extend to the lower bronchial passages. The bacterial flora present in the broncho-pneumonic lesions therefore varies considerably, but the pneumococcus and influenza bacillus are predominant and are often present together. In very severe influenza, such as occurred in 1918, in addition to broncho-pneumonia, lobar pneumonia due to the pneumococcus occurred, and sometimes even a true septicæmia due to this organism—an indication of remarkably lowered resistance. We may note that empyema due to the pneumococcus is more frequent in children than in adults, being a not uncommon sequel of broncho-pneumonia.

It is difficult to explain why sometimes the pneumococcus is associated with a spreading inflammation as in lobar pneumonia, while at other times it is chiefly localised in the catarrhal patches in broncho-pneumonia. It is quite likely that in the former condition the organism is possessed of a different order of virulence, and probably the susceptibility of the lung tissue varies at different periods of life—the resulting lesion, of course, depends on both of these factors. We have, however, a closely analogous fact in the case of erysipelas, this being a spreading lesion produced by a streptococcus, which when less virulent, causes only local inflammatory and suppurative conditions. As already mentioned, the existence of a crisis in lobar pneumonia, indicating a rapidly developed immunity, is a characteristic feature, and the association of this with a rapid growth of the organisms throughout the framework of the lung has undoubtedly an important significance. When the organisms are mainly present within the air vesicles and bronchioles, as in broncho-pneumonia, this rapid immunity reaction is not met with.

In children the pneumococcus may extend along the Eustachian tubes and give rise to otitis media, this being a fairly frequent and important lesion; thence it may spread to the brain and give rise to leptomeningitis. It may be noted that next to the lungs the meninges are the parts most liable to

attack by the pneumococcus, usually secondary to a form of pneumonia or otitis media, but sometimes as a primary occurrence (p. 361). Other primary pneumococcal lesions (apart from pneumonia), such as arthritis, peritonitis, etc., are also met with. Primary pneumococcal peritonitis affects mainly young female subjects in whom infection reaches the peritoneum from the vagina (McCartney). Not infrequently rhinitis is due to the pneumococcus as are also inflammatory and suppurative lesions in the maxillary antrum and frontal sinus. Conjunctivitis is sometimes caused by the pneumococcus (p. 316), as is also corneal ulcer.

Immunity to Pneumococcal Infection.—Lobar pneumonia represents an inflammatory lesion accompanied by the symptoms of an acute poisoning. In very few cases does death take place from the functions of the lungs being interfered with to such an extent as to cause asphyxiation; it is through cardiac failure, from great interference with the heat-regulating mechanism, and from general nervous depression that death usually results. These considerations, taken in connection with the fact that the organisms are found in the greatest numbers in the lung, suggest that a toxic action is at work. We may say, however, that attempts to separate specific soluble toxins have failed, and that the poisonous substances are apparently of the type of endotoxins set free by lysis and of similar nature to those formed by various other bacteria. In conformity with this, it is found that while active immunity can be readily obtained, there is no evidence that it depends upon specific antitoxins; it is *antibacterial*, and is due to the development of powers to destroy the invading organisms.

Animals can be immunised against the pneumococcus by inoculation with virulent cultures killed by heating at 55° C., with autolysed cultures or with cultures which have become attenuated in various ways. Sometimes one or two injections, at intervals of several days, are sufficient for immunisation, but the immunity has been observed to be usually of a fleeting character and may not last more than a few weeks; a process of intensive and rapid immunisation is described below. The serum of such immunised animals neutralises the action of pneumococci in susceptible animals when added to the organisms; it protects also against subsequent inoculation with a certain dose of pneumococci, and if injected within twenty-four hours after inoculation, may even prevent death.

Pneumococcus infection is, however, one of the conditions where a more effective immunity is developed by inoculating with

living than with dead organisms. Neufeld and Rimpau insisted that the ultimate use of living virulent cultures was necessary for the attainment of a high degree of immunity. Blake and Cecil in their work on experimental pneumonia in monkeys likewise found that small doses of living virulent pneumococci or larger doses of the same organisms in an attenuated state protected against intratracheal injection of virulent pneumococci, while vaccines consisting of dead organisms failed to do so. The latter, however, modified the secondary invasion of the blood by pneumococci when pneumonia developed, and also afforded protection against experimental pneumococcal septicæmia. There was thus a humoral immunity without protection against intratracheal infection. They point out, however, that these results do not mean that dead vaccines may not protect the human subject against pneumonia under natural conditions, seeing that the monkey is more susceptible. As a matter of fact, we have evidence that dead culture vaccines do afford a degree of protection. The use of the living virulent organism is, of course, difficult to control, and is impracticable as a preventive measure against natural pneumonia in the human subject.

The protective potency of an anti-pneumococcal serum as measured by the number of fatal doses of virulent pneumococci against which a given amount of serum will protect an animal (*e.g.* a mouse), may reach a high degree. In studying the development of antibodies in the blood, Neufeld and Händel found that there may be little antagonistic effect till a certain concentration of these is reached, while beyond this point rapid destruction of a large number of pneumococci may occur. Armstrong has studied quantitatively the development of pneumococcal antibodies and has found, for example, after a single dose of dead vaccine, that there is an inductive phase of about three days, following which for two or three days there is an access of antibodies to the blood at a rapidly increasing rate, corresponding to a geometrical progression. The increase then becomes less rapid and the maximum concentration occurs about the eighth day. He finds further that in a case of lobar pneumonia, running an ordinary favourable course, similar phenomena are met with. A rapid outpouring of antibodies occurs about the fifth day, and the content in the serum increases till about the time of the crisis, after which it remains high for some time. We thus have the picture of a rapid development of active immunity, which on reaching a certain point results in a rapid destruction of the organisms.

An anti-pneumococcal serum exhibits various antibody reactions, including agglutination and precipitation (p. 223). There is no evidence, however, that an antiserum, in virtue of its immune-bodies, possesses bactericidal action. The organism is sensitive, however, to opsonic action, and thus an antiserum may exert its beneficial action by means of its opsonins (or bacteriotropins). Observations have been carried out on the opsonic properties of the serum in cases of pneumonia, and it has been found that towards the time of crisis in favourable cases the opsonic index rises distinctly, and after defervescence gradually returns to normal. Blake and Cecil in their work on experimental pneumonia in monkeys did not consider it possible to explain the immunity by the antibodies demonstrable in the serum, and considered that some other factor, such as changes locally in the tissues, might be concerned in the immunity reaction and the destruction of the organisms. Recently Sia, Robertson and Woo have shown that the immunity acquired during the illness is associated with an increased power of the serum to promote the killing of pneumococci by a rabbit serum-leucocyte mixture. At the crisis they found this property markedly developed, and absent in fatal cases. These results tend to emphasise the importance of opsonic antibodies in immunity to the pneumococcus. According to H. D. Wright's observations on immunised rabbits, the property possessed by the whole blood of delaying growth of virulent pneumococci *in vitro* is the manifestation of antibody activity which appears earliest and lasts longest.

The leucocytosis in pneumonia has long been recognised, and when present in distinct degree indicates that a satisfactory cellular response is taking place. It, however, scarcely indicates more than this, and certainly does not promise recovery, as death may occur in a variety of ways. On the other hand, the absence of leucocytosis, or its disappearance after being present, is undoubtedly of unfavourable omen.

It may be noted here, in conclusion, that in man immunity against pneumonia may be short-lived, as in many cases of pneumonia a history of a previous attack is elicited. It is, of course, possible that a second attack may be due to a type of pneumococcus differing immunologically from that of the first infection (*vide infra*). If this were so, any immunity persisting from the earlier disease would not be protective, as the immunity mechanism is specific for each type of the organism.

Differentiation of Types of the Pneumococcus by Antisera.—The possibility of effecting this is one of the most im-

portant consequences of the study of immunity against the pneumococcus. It had been long recognised that strains of the pneumococcus derived from different sources present individual peculiarities, but it was not till the exhaustive investigation of the subject in the Rockefeller Institute, New York, that definite results were obtained. In the study of the agglutinating and protecting properties of antisera prepared by inoculating animals against a long series of cultures isolated from cases of acute lobar pneumonia, it was proved that sera derived from certain strains, on the one hand, would almost indiscriminately agglutinate some of these strains, and, on the other, had little or no effect on other strains. It was further found that the agglutinating and protective qualities of these sera were parallel. In this way it was possible to group the strains into four types. Three of them (I., II., III.) were serologically well defined, and a fourth (IV.) was formed of strains in which an antiserum usually agglutinated only the strain which originated it, and had little or no capacity of agglutinating the strains of Types I., II., III. The members of Type III. could be recognised not only by their originating agglutinating sera specific to the type, but presented cultural features which characterised them as the *Pneumococcus mucosus* (see p. 331). Types I. and II. together accounted for 60 per cent. of the cases of pneumonia studied and were of relatively high virulence for man, this being specially the case with Type II. Type III., while accounting for only 12 per cent. of cases, was of highest virulence, the mortality with it being 45 per cent. Type or Group IV. was found in 24 per cent. of cases and caused the lowest mortality (16 per cent.); the strains occurring in the mouths of healthy individuals were found to belong mainly to this type. It is probable, however, that in different parts of the world different types prevail. Thus, in South Africa, Lister found that, while the New York Types I. and II. were common, nearly a third of all cases of pneumonia were associated with another type which apparently did not occur to any extent in New York. Davidson and McLachlan found that among a variety of pneumococcal infections (studied in Edinburgh in 1924–25) Type I. predominated and was present in 64 per cent. of all cases, while Type II. was relatively infrequent (7 per cent.). Type III. and Group IV. represented respectively 15 per cent. and 14 per cent. of the infections. It is interesting to note that the type-incidence may vary at different times in the same area: thus in Edinburgh during 1929–30 Type II. was the most prevalent variety, causing 40 per cent. of cases of lobar pneumonia,

while Type I. occurred in 29 per cent. The death-rate in Types I. and II. cases respectively was 23 and 31 per cent. (Alston and Stewart). Types I. and II. are apparently more invasive than Type III. or Group IV. (R. Cruickshank) as illustrated by the prevalence of I. and II. in primary meningitis, peritonitis and empyema, and the frequency of the others in otitis media, secondary meningitis and conjunctivitis.

The constituent serological types of Group IV. have been classified by Cooper and her co-workers in America. Twenty-nine types have been identified (designated IV. to XXXII.), and only a small proportion of strains remained unclassified. Certain types were found to be more prevalent than others in lobar pneumonia of adults. The majority of the types were also recognised in normal persons and in other infections of the respiratory tract. This heterogeneous group is largely responsible for primary broncho-pneumonia, the infection being an endogenous one (R. Cruickshank). Blacklock and Guthrie in a study of lobar and broncho-pneumonia among infants and children found that 90·7 per cent. of pneumococcus strains from these conditions belonged to Group IV.

The Specific Substances of the different Types of Pneumococcus.—In 1917 Dochez and Avery noted in the blood and urine of cases of lobar pneumonia a substance which gave a precipitation reaction with an antiserum for the type of pneumococcus causing the infection. It could be detected by mixing equal quantities of clear centrifuged urine and an appropriate antiserum. The appearance of this substance in the urine depends on the severity of the case, and a progressive increase in amount is significant from a prognostic standpoint. It was observed also in young cultures, and apparently diffuses readily from the organisms into the surrounding medium. Heidelberger and Avery isolated this specific substance from Type II. pneumococcus. They used a method of fractional precipitation by alcohol, acetone and ammonium sulphate, and dialysis. It appeared to be a polysaccharide yielding 79 per cent. of reducing sugars on hydrolysis, and gave no protein reactions. Though it did not act as an antigen *in vivo*, it was able to react in a specific manner *in vitro* with a homologous antipneumococcal serum. In its immunological relations it belongs to the group of "haptens" (Landsteiner). An immunologically and chemically identical polysaccharide has been demonstrated in yeast and in one serological type of the pneumobacillus (p. 349). Similar polysaccharides were later obtained from Type III. and Type I. pneumococci, but all proved to be chemically different substances, each reacting specifically with the corresponding antiserum. The precipitin reactions elicited with these bodies occurred sometimes in very high dilutions. The Type I. substance was found to contain nitrogen as part of its molecule. These specific substances have been extensively studied by various workers from the chemical and immunological standpoints, and the facts suggest that their

formation is intimately related to the capsulation and the virulence of the organisms. Strains which have undergone "rough" variation and lost virulence also show a loss of their capacity to form their specific carbohydrate. Associated with these changes there is an absence of type-specific characters and capsule formation These carbohydrate substances also produce cutaneous reactions in specifically immunised persons or animals. In addition to this type-specific polysaccharide "hapten," pneumococci also contain a protein antigen which is not type-specific but common to all types; thus, avirulent "rough" variants lacking the type-specific "hapten" contain this protein antigen. A species- or group-specific polysaccharide has also been isolated.

It has been shown by Sia that the specific substance added to a serum-leucocyte mixture annuls the bactericidal effect of the latter. This effect has also been found to be specific; thus, the soluble carbohydrate of Type I pneumococcus interferes specifically with the bactericidal action of whole-blood towards this type. This property may be an important factor in pneumococcal infection, and the specific polysaccharide of the organism may possess "aggressin" functions. It is not *per se* toxic. The specific effect on the bactericidal power of whole-blood may be due to its union with and fixation of opsonic substances. The transformation of one type of pneumococcus to another type by inoculating its "rough" derivative along with a dead culture of the latter type has been recorded by Griffith and by Dawson. Thus an "R" variant may acquire the power to form a polysaccharide specifically different from that possessed by the original strain According to recent observations by Alloway, an R variant derived from a type-specific S form may be changed *in vitro* by growth in broth containing anti-R serum along with a heated filtered extract of an S-form pneumococcus of different type, and thus becomes a virulent S-form organism identical in type with the bacteria from which the extract was derived. These results are of great biological interest.

In addition to the type-specific polysaccharides referred to above, various other non-protein substances have been separated which may enter into the antigenic composition of the pneumococcus.

Avery and Dubos have discovered an enzyme extracted from a saprophytic bacterium which is capable of hydrolysing the capsular carbohydrate of the pneumococcus. Type III pneumococcus acted on by this enzyme is rendered avirulent and the enzyme has been found to exert a protective action *in vivo* in mice against infection by this organism.

Methods of classifying Pneumococci by Agglutination.—Specific agglutinating antisera for the three types respectively (I., II., III.) are required. A white mouse is inoculated intraperitoneally with 0·5 to 1 c.c. of a saline emulsion of a bean-sized piece of sputum, preferably quite fresh, freed from surface contamination by washing in sterile saline. The mouse may die in from five to twenty-four hours, and if the peritoneal exudate contains a strong and fairly pure growth of the pneumococcus the abdominal cavity is washed out with 5 c c. saline, cultures being at the same time made in broth and on blood-agar plates. The peritoneal washings are first centrifuged slowly to precipitate gross material, and the supernatant fluid is then centrifuged at a high speed to precipitate the bacteria.

The bacterial deposit is suspended in saline to form a fairly heavy suspension which is used for a microscopic sedimentation test. If the pneumococci in blood cultures or in exudates are to be employed, suspensions may be obtained by similar procedures. A bacterial emulsion being prepared, 0·5 c.c. of Serum I. (1 : 20), 0·5 c.c. of Serum II. (undiluted), 0·5 c.c. of Serum II. (1 : 20), and 0·5 c.c. of Serum III. (1 : 5) are placed in four tubes, and 0·5 c.c. bacterial emulsion added to each, and in a fifth tube a mixture of 0·1 c.c. sterile ox bile and 0·4 c.c. bacterial suspension is made up; the series is placed in a water bath or incubator at 37° C. for one hour, and the result read. Sedimentation in any one of the four tubes indicates that the strain belongs to the type by the serum of which it is agglutinated; if no reaction occurs in any of the tubes, and the organism is soluble in bile, it is classified as Group IV. The serum dilutions stated are generally suitable for the test, but it is advisable to ascertain the optimal dilutions of antisera by trial with known strains of the three types.

Without waiting for the death of the mouse, a small amount of the peritoneal fluid may be withdrawn by means of a blunt needle three to six hours after the injection of sputum. Four separate small drops of the fluid are expelled from the needle on to a slide: the first drop is mixed with a loopful of saline and spread into a thin film as a control; the others are mixed with a loopful of 1 : 10 dilutions of antisera to Types I., II., and III. pneumococci respectively and are similarly spread. The films are fixed and stained and examined with the immersion lens. Agglutination occurring only in the presence of one of the antisera indicates the type of the organisms. If the results are inconclusive, the animal is available later for examination as described above (Sabin)

Direct typing of pneumococci.—As shown originally by Neufeld the pneumococcus in sputum undergoes a distinct change observable microscopically when it is acted on by specific serum; this consists in a visible "swelling" of the capsular zone which then appears wide and refractile. By means of this reaction the type present in a case can be ascertained directly and quickly without animal inoculation. The sputum is emulsified in normal salt solution and successive drops of the preparation are mixed on slides with drops of Types I., II., and III. antisera (from rabbits). A control included in the test consists of a drop of the emulsified sputum mixed with a drop of saline. Each mixture is covered with a cover slip and examined microscopically with a $\frac{1}{12}$ inch lens. The observation of the specific change referred to above is aided by lowering the condenser and reducing the aperture of the diaphragm. The reaction is complete in two or three minutes. Observations may also be facilitated by adding a drop of Löffler's methylene-blue to the mixtures; the swollen capsules appear unstained and have a ground-glass appearance (Sabin).

The Treatment of Pneumonia with Antisera.—Many years ago the Klemperers treated a certain number of cases of human pneumonia by serum derived from immune animals, apparently with a certain measure of success, and afterwards Römer issued through Merck a polyvalent serum prepared by immunising different species of animals with growths of the pneumococcus

on sheep-serum-glycerol-broth and mixing their sera. The results obtained, though in some cases satisfactory, were irregular, and Neufeld and Händel then insisted that it was essential that the antiserum used should correspond to the particular strain present in the case to be treated.

Evidence confirming this view was obtained in the Rockefeller Institute investigations on pneumonia, in which the determination of the different types of the pneumococcus was followed by an estimate of the therapeutic capacities of the antisera prepared against Types I., II., III. (*vide supra*). It was found that while the antiserum to Type I. had a curative effect on cases of pneumonia due to the Type I. pneumococcus, the antisera to Types II. and III. had practically no effect on cases attributable to these types; furthermore, the antiserum to Type I. had little or no effect on cases caused by Types II. and III. These facts threw light on the irregular and generally disappointing result obtained with the ordinary polyvalent anti-pneumococcal sera.

The Rockefeller Institute serum was prepared by immunising horses first with dead cultures; daily injections were given for six days, followed by an interval of a week, then six further daily injections were given; it was sometimes necessary to follow these up by the use of living organisms. Uniformity of strength in successive sera thus prepared was secured by determining the largest amount of an eighteen hours' culture against which 0·2 c.c. of the serum would protect a white mouse—a comparison with the effects of the same amount of a standard serum being at the same time made.

In the therapeutic application of the serum in a case of Type I. pneumonia large quantities had to be used, and it was therefore a necessary preliminary to determine whether the patient exhibited supersensitiveness to horse serum, and to desensitise him if this existed (*vide* p. 272). The initial dose was from 90–100 c.c. given intravenously, and the injections were repeated every eight hours till about 250 c.c. serum had been given. Very soon after commencement of treatment the temperature often rose, but in many cases this was quickly succeeded by a fall, with improvement in the patient's general condition, stoppage of extension of the lung lesion, and prevention of invasion of the blood by the pneumococci. The effects of the treatment proved satisfactory—of 107 cases treated in the Rockefeller Institute Hospital up to October 1917 only 7·5 per cent. died, as compared with a mortality of 25 to 30 per cent. in cases of Type I. pneumonia before the serum treatment was introduced.

Recently attempts have been made to develop the serum-therapy of acute pneumonia by the use of polyvalent or mono-

valent antisera concentrated and "refined" by Felton's method, in which the antibody-containing globulins are precipitated by dilution with distilled water or weakly acid buffer solutions. This enables an effective dose to be given intravenously in relatively small bulk and with less frequent and marked reactions due to the protein injected (Park, Bullowa and Rosenblüth). The concentrated serum is stated to have five to ten times the potency of the unrefined serum. A single dose is 10,000 Felton units (*vide infra*). The results indicate a pronounced efficacy of the serum in Type I. infections; in Type II. cases the therapeutic effect is apparently less marked; infections by other types have not been appreciably benefited. It must be emphasised that an essential factor in determining the value of serum treatment is the stage of the infection in which it is commenced: early administration is essential. In view of the therapeutic limitation of antisera to Types I. and II. pneumococci, a bivalent concentrated serum for these types has been used during recent years. The results with this serum indicate a definite therapeutic effect in cases due to Type I. pneumococcus, the severity of the illness being lessened, the febrile period shortened, and the mortality reduced. In cases due to Type II. the efficacy of the serum has been less pronounced. It seems preferable to use monovalent antisera and if the clinician desires to inject both Type I. and II. sera at an early stage, before the organism has been typed, the two sera can be mixed and administered in one dose (O'Brien).

Standardisation of Anti-pneumococcus Serum.—In general the basis of standardisation has been the protective value of the serum against virulent pneumococci injected into mice, the quantitative tests being made by mixing varying dilutions of a pneumococcus culture with a given quantity of serum, or varying quantities of serum with a given number of pneumococci. The latter is the system adopted by Felton whose unit of potency is the amount of serum which protects a mouse against a million fatal doses of pneumococcus culture. In the League of Nations' method a constant volume of broth culture (0·001 c.c.) is mixed with decreasing volumes of serum (0·001 c.c., 0·0005 c.c., 0·00025 c.c., etc.) and after five minutes the mixtures are injected intraperitoneally. This system has been examined by Parish and by Trevan and satisfactory titrations have been obtained, the error of the test depending on the number of animals used (see Trevan). The titration of a new serum should be carried out by comparison with a standard serum.

Therapeutic mechanism of anti-pneumococcal serum.—The serum exerts a definite protective action against the pneumococcus under experimental conditions, the effect being type-specific. This has been generally interpreted as due to its specific action in sensitising the organisms to phagocytosis. Robertson, Sia and Cornwell

have found, however, that a therapeutic serum does not promote the bactericidal action by leucocytes (rabbit) unless in the presence of fresh serum (rabbit). The pneumococcus does not produce exotoxin in culture and there is no evidence as to whether antisera possess antitoxic properties. The therapeutic mechanism of an antiserum is apparently a complex one and is possibly dependent on other factors besides opsonic or bacteriotropic action.

Prophylactic Vaccination.—In the South African mines a special situation existed in consequence of the great susceptibility to pneumonia occurring in the native labourers, who are chiefly recruited from subtropical regions. As the case incidence ran from 30 to 150 per thousand per annum, and the mortality from 10 to 30 per thousand, the disease became a very serious problem. Almroth Wright introduced prophylactic vaccination, and Lister, founding on his investigations (*vide supra*), prepared a vaccine containing the three prevalent types of the pneumococcus. Three injections at seven-day intervals of, in all, 7000 million cocci, killed by an antiseptic, were administered A very marked diminution in the incidence of the disease followed. Cecil and Austin in the United States record the results of vaccinating about 40 per cent. of 32,000 men in a camp with a mixture of Types I., II , and III., two to four doses being given. In the subsequent period of ten weeks no cases of pneumonia due to these types developed among those vaccinated, while among the unvaccinated there were twenty-six cases.

Methods of Examination.—In stained films of sputum, pus, or other exudate containing pneumococci, the outstanding feature is the predominance of diplococcal forms, the elements of which may have a lanceolate shape and which are Gram-positive. Often a capsule stain demonstrates the capsule in such material, and it may even appear stained in Gram films. Cultures on blood-agar (preferably heated-blood-agar) should be made which after twenty-four hours at 37° C. will, if the pneumococcus be present, show characteristic colonies. Bile-solubility and the reaction with inulin may be tested ; a mouse may be inoculated intraperitoneally to test the pathogenicity and to afford in blood films corroborative evidence of capsulation. The serological type of the pneumococcus can also be determined by the methods described above.

Particularly in early cases of pneumonia sputum may not be obtainable for bacteriological examination and for the typing of the organism. By puncture of the affected lobe in a majority of such cases material can be obtained which yields a pure culture of the organism present (Stewart). An exploratory needle of fine bore about 10 cm. in length attached to a 20 c.c. Record syringe is used. Where the lower lobe is affected the needle is inserted through the seventh, eighth, or ninth intercostal space posteriorly in the mid-scapular line. Preliminary local anæsthesia may be induced at the selected site. The

Exploratory needle is inserted directly into the lung substance for 8 to 10 cm. and a few drops of blood-stained fluid are aspirated. The fluid is mixed with 5 c.c. of broth and part of this can be utilised for inoculation of a mouse.

The Pneumobacillus of Friedländer (*Klebsiella pneumoniæ*).—This organism is of historic interest, as it was the first organism described in pneumonia, though there is little doubt that in early days it was often confused with the pneumococcus. It does not occur alone in more than about 1 per cent. of cases of pneumonia; in the pneumonia caused by it the consolidated lung has often a somewhat slimy or mucoid character. In the sputum it may appear as a very short diplobacillus (1 to 2 $\mu \times 0{\cdot}5\ \mu$) possessing a capsule, but it is also frequently seen in the form of long rods. It stains by ordinary methods, but is Gram-negative. It is non-motile and non-sporing.

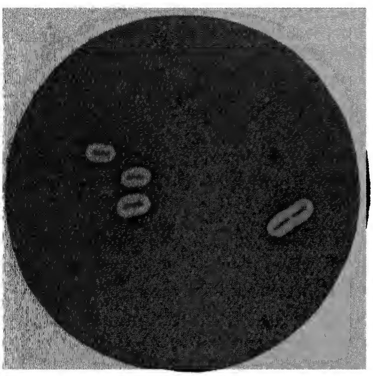


FIG. 51.—Friedlander's pneumobacillus; film from a culture. Eosin capsule method. $\times 10000$

It is an aerobe and can be easily isolated on agar plates, on which it forms large whitish moist viscid colonies (cf. *B. lactis aerogenes* of *B. coli* group). In gelatin stabs, at the site of the puncture the growth is sometimes heaped up above the level of the medium, and along the needle track there is a white granular appearance. The gelatin is not liquefied. The organism grows well on all ordinary media, and on these its bacillary nature is in marked evidence (Fig. 51). It may form capsules in serum broth. There is some variation among different strains as regards their fermentative reactions. Certain strains conform in their biochemical characters to the inosite fermenting sub-group of the coliform bacilli (*vide* p. 514 and table p. 556) and ferment glucose, lactose, saccharose, adonitol, and inosite, usually with gas production. Some strains, however, fail to ferment lactose. Formation of indole from peptone is variable. The same applies to the Voges-Proskauer and Methyl-red reactions (*vide* p. 510). It might be said, however, that the majority of strains yield a negative indole reaction and a positive methyl-red reaction. The pneumobacillus is closely related to the *B. coli* and it is practically impossible to draw any distinction between these capsulated Gram-negative bacilli from the respiratory tract and similar forms occurring in the intestine (e g. *B aerogenes*).

By serological reactions with antisera, it has been shown that strains of this organism from cases of pneumonia can be classified into three types (A, B, and C) and a fourth heterogeneous group. As in the pneumococcus, specificity depends on a carbohydrate

substance associated with the capsule, and it is of special interest that Type B is serologically related to pneumococcus Type II. (Julianelle).

When injected into mice and guinea-pigs it causes a septicæmia and can be seen in the heart blood to possess a capsule. It is less pathogenic to rabbits and dogs, but when injected into the trachea in these animals it originates a pneumonia. Type C is apparently of less virulence than Types A and B. As stated above, it is the only organism present in a small number of cases of human pneumonia, and it has also been isolated from conditions of empyema, meningitis, appendicitis, and pyæmia; organisms closely related have been found in ozæna and rhinoscleroma (*vide infra*). It is a not infrequent inhabitant of the mouth and nose of healthy individuals.

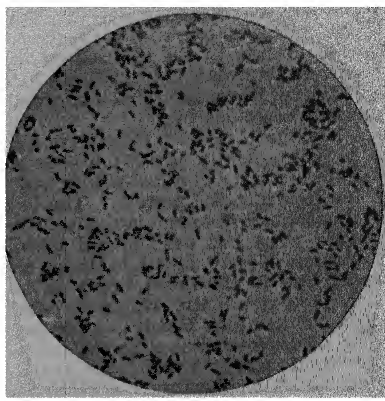


FIG. 52.—Friedländer's pneumobacillus,[1] from a young culture on agar, showing some rod-shaped forms.
Stained with thionin. ×1000.

The Bacillus of Rhinoscleroma (*Klebsiella rhinoscleromatis*). — This organism is considered here as it is biologically related to the pneumobacillus. Rhinoscleroma belongs to the group of infective granulomata. It is characterised by the occurrence of chronic nodular thickenings in the skin or mucous membrane of the nose, or in the mucous membrane of the pharnyx, larynx, or upper part of the trachea. The nodules are of considerable size, sometimes as large as a pea; in the earlier stages they are comparatively smooth on the surface, but later they become shrunken and the centre is often retracted. The disease is scarcely ever met with in this country, but has been reported on the Continent, especially in Austria and Poland. In the granulation tissue of the nodules there are to be found numerous round and rather large cells, which have peculiar characters and are often known as the cells of Mikulicz. Their protoplasm contains a collection of somewhat gelatinous material which may fill the cell and push the nucleus to the side. Within these cells there is present a characteristic bacillus, occurring in little clumps or masses, chiefly in the gelatinous material. A few bacilli also lie free in the lymphatic spaces around. This organism, known as the bacillus of rhinoscleroma, was first observed by Frisch. The bacilli have the form of short oval rods, which, when lying

[1] The apparent size of this organism, on account of the nature of its sheath, varies much according to the stain used. If stained with a strong stain, *e.g.* carbol-fuchsin, its thickness appears nearly twice as great as is shown in the figure.

separately, can be seen to possess a distinct capsule; in all their microscopical characters they correspond closely with Friedländer's pneumobacillus. They are Gram-negative, and as a rule they are the only organisms demonstrable.

From the affected tissues this bacillus can be easily cultivated by the ordinary methods. In the characters of its growth in the various culture media it presents a close similarity to the pneumobacillus, as it does also in its fermentative actions. Minor differences have been described, but it is doubtful whether any distinct line of difference can be drawn between the two organisms.

The serum of patients suffering from the disease gives fixation of complement when tested with an emulsion of the bacillus, but varying results have been obtained as regards the validity of the test in the differentiation of the bacillus from the allied organisms.

The evidence that the organisms described are the cause of this disease consists essentially in their constant presence and their special relation to the affected tissues, as already described. Experimental inoculation has thrown little light on the subject, though one observer has described the production of nodules in the conjunctivæ of guinea-pigs.

A bacillus possessing closely similar characters has been found to be very frequently present in ozæna, and is often known as the *Bacillus ozænæ*. The last-mentioned organism is said to have more active fermentative powers. The bacillus of Perez which has also been described in ozæna is referred to on p. 321.

From what has been stated it will be seen that a number of organisms, closely allied in their morphological characters, have been found in the nasal cavity in healthy or diseased conditions. There is no doubt that rhinoscleroma is a specific disease with well-marked characters, and it is quite possible that one member of this group of organisms may be the causal agent, though indistinguishable from others by culture tests. Recent investigators consider the "bacillus of rhinoscleroma" to be identical with the pneumobacillus, and its presence in the affected tissues to represent merely a secondary invasion (Perkins).

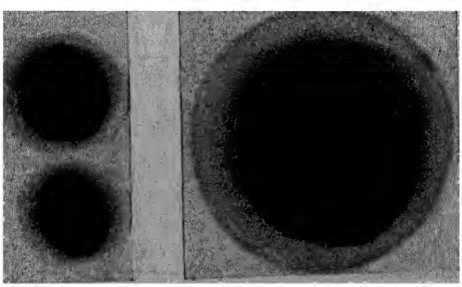
The Meningococcus and Epidemic Cerebro-Spinal Meningitis (or Cerebro-Spinal Fever)

Since the beginning of the nineteenth century the occurrence of epidemics of cerebro-spinal meningitis has been recognised. As the result of observations on this disease in different parts of the world, it has been established that the causal agent is the *Diplococcus intracellularis meningitidis*, first described by Weichselbaum, and usually known as the *Meningococcus*. Epidemics are met with in military barracks among young recruits. In the civil population cases occur chiefly from infancy to young adult age, while the sporadic form of posterior basal meningitis among young children is also due to this organism.

The Meningococcus (*Neisseria meningitidis*) is a small coccus measuring about 0·8 to 1 μ in diameter; it usually occurs in

pairs, the adjacent sides being somewhat flattened against each other. In many cases the cocci are chiefly contained within polymorphonuclear leucocytes in the exudate (Fig. 53); sometimes, however, the majority may be lying free, and this is especially the case in virulent infections. It stains readily with basic aniline dyes, but is Gram-negative. It may be noted that the cocci frequently show metachromatic granules demonstrable by staining with Löffler's methylene blue and also by Neisser's method as applied to the diphtheria bacillus (p. 375). Both in appearance and in its staining reactions it is closely similar to the gonococcus (p. 363). The organism can readily be cultivated aerobically outside the body, but the

FIG. 53.—Film preparation of exudate from a case of meningitis, showing the meningococci within leucocytes. See also Plate I., Fig. 3.
Stained Gram's method and dilute carbol-fuchsin.



a *b*

FIG. 54.—*a*. Two-days' colonies of the meningococcus, ×9; *b*. the same, in which illumination has been arranged to show finely granular centre and transparent margin. ×12. Compare with Fig. 57.

From photographs by the late Prof. W. B. M. Martin.

conditions of growth are somewhat restricted—agar with an admixture of serum, ascitic fluid, or blood (p. 69) or

boiled-blood-digest-agar, is to be recommended. Growth does not occur usually on ordinary media without serum. The optimum reaction is about pH 7·5. Growth takes place best at the temperature of the body, and practically ceases at 25° C. On these media the colonies are circular discs with a slightly opaque centre fading into a delicate transparent margin (Fig. 54), and they have a smooth, shining surface; they have a slightly mucoid consistence and readily emulsify in water or normal saline. When examined under a low magnification the centre appears somewhat yellowish, and the margins usually are smooth and quite regular; at a later period of growth slight crenation may appear, especially when the medium is somewhat dry. The colonies may be of considerable size, reaching sometimes a diameter of 2 to 3 mm. on the second day. Variations in appearance are met with and dissociation may occur with the development of R (rough) and other variant forms. In serum-broth the organism produces a general turbidity with formation of some deposit after a day or two.

In the preparation of a medium suitable for culture purposes most attention has been directed to the readiness with which growth takes place and to the abundance of the growth, but, as pointed out by Murray and Ayrton, retention of the virulence of the organism should also be a criterion of suitability. They have found that retention of virulence depends upon substances contributed by the digest used as a basis, and they have also made the interesting observation that the addition of an extract of polymorphonuclear leucocytes raises the virulence *in vitro*. (For the formulæ of their media their original papers must be consulted.) It has been shown also that, according to the medium used, the virulence may be lowered and raised several times in succession.

The meningococcus ferments glucose and maltose with acid production, properties which distinguish it from *Micrococcus catarrhalis* (*vide infra*); and it has no action on lactose and saccharose. Fermentation tests can be carried out by means of either fluid or solid media containing serum and 1 per cent. of the sugar to be tested, along with neutral-red or litmus as an indicator. In cultures the organism presents the same appearance as in the body, and often shows tetrad formation. There is also a great tendency to the production of involution forms (Fig. 55), many of the cocci becoming much swollen, staining badly, and afterwards undergoing disintegration. This change, according to Flexner's observations, would appear to be due to the production of an autolytic enzyme, and he has also found that this substance has the property of producing

dissolution of the bodies of other bacteria. The life of the organism in cultures is a comparatively short one; after a few days cultures will often be found to be dead, but by making subcultures every three or four days strains can be maintained alive for considerable periods. Cultures survive longer at 37° C. than at room temperature. On egg medium, however, it survives for a considerable time. The organism is readily killed by heat at 55° C., and it is also very sensitive to weak antiseptics; drying for a few hours has been found to be fatal to it. The facts established accordingly show it to be a somewhat delicate parasite.

As stated above, the organism occurs in the exudate in the meninges and in the cerebro-spinal fluid, and it can usually be obtained by lumbar puncture. In acute cases, especially in the earlier stages, it is usually abundant; but in the later stages of cases of more subacute character, its detection may be a matter of difficulty, and only a few cocci may be found after a prolonged search. But it should be recognised that *at any stage* microscopic examination and even cultivation may sometimes give a negative result. In most cases the lesions are practically restricted to the nervous system, but occasionally complications occur, and in these the organism may be present. It has been found, for example, in arthritis, pericarditis, pneumonic patches in the lung, and in other inflammatory conditions associated with the disease, and also occasionally in purpuric patches in the skin and stomach, though the ordinary petechial eruption seems to be of toxic origin. It may be obtained from the blood during life; occasionally such cases may recover.

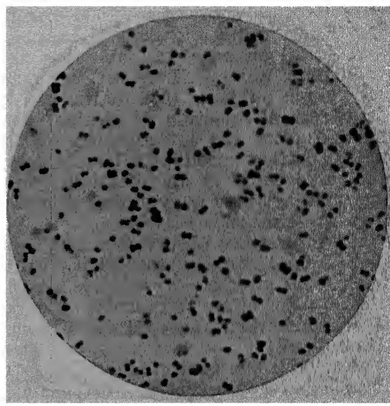


FIG. 55.—Pure culture of meningococcus, showing involution forms. From a 48 hours' culture. Stained by Gram's method and dilute carbol-fuchsin. ×1000.

Experimental inoculation shows that the ordinary laboratory animals are relatively insusceptible to this organism. An inflammatory condition may be produced in mice and guinea-pigs by

intraperitoneal injection, and a fatal result with symptoms of collapse may follow. The results vary according to the virulence, but often the organism does not undergo very active multiplication, though it may sometimes be cultivated from the blood ; none of the lesions in the nervous system are reproduced. Similar results are produced by the endotoxin in dead cultures, and occasionally the lethal dose of the dead organisms may equal that of the living (Gordon). There is thus evidence that an active toxin plays an important part in the pathology of the disease. Flexner and also Stuart McDonald have shown that cerebro-spinal meningitis may be produced in monkeys by injections of the organism into the spinal canal, the latter observer finding that exudate containing meningococci was more effective than cultures. In such experiments the organisms extend upwards to the brain, and produce meningitis within a very short time. The resulting lesions, both as regards their distribution and general characters, and also as regards the histological changes, resemble the disease in the human subject. Even these animals, however, are manifestly less susceptible than the human subject. Meningitis has been produced in rabbits by intracisternal injection of virulent meningococci ; a similar effect resulted from the introduction of filtered suspensions, and even boiled culture produced histological changes in the meninges similar to those in an infective meningitis (Branham and Lillie). Such findings may be regarded as manifestations of the toxic action of the organisms.

The interesting observation has been made by Miller that even a small dose of meningococci if suspended in a solution of gastric mucin can produce a rapidly fatal infection on intraperitoneal injection in mice. Moreover, the animals show a pronounced general infection, the organisms being demonstrable in the circulating blood.

The claim has been made recently that the meningococcus produces an exotoxin (Ferry, Norton and Steele). It has been stated that when grown in flasks in hormone broth (pH 6·6) a pellicle forms and within a few days a soluble toxin can be demonstrated which in high dilutions produces skin reactions in animals and which incites antitoxin formation. Ferry has used this antitoxin in therapeutic tests in infected monkeys and states that it is effective in preventing a fatal result. According to Malcolm and White it is possible to extract a highly toxic substance from cultures but they regard it as an endotoxin. Other workers have also found that an antiserum in high dilution may neutralise certain toxic preparations obtained from the meningococcus. The high toxicity of the organism is undoubted and there is some evidence that the toxin is antitoxinogenic. It is difficult to make any statement as to whether this product should be classified as an endo- or exo-toxin.

Infection.—The meningococcus can usually be found in the naso-pharynx of patients suffering from the disease, and there is no doubt that this is the usual channel of invasion, infection being conveyed by secretion droplets from the mouth, throat, and nose. In cases where recovery occurs, the organism may persist there

for a varying period of time—usually only for a week or two, but sometimes for months. There is difference of opinion as to the route by which the organism passes from the naso-pharynx to the meninges. One view is that it passes directly by the lymphatics to the base of the brain, but satisfactory evidence of this is wanting. The other view is that it passes by the blood stream; this is in accordance with what occurs in other infections, and is also supported by the fact that in some very acute cases with purpuric eruption, it has been found in the blood before meningeal symptoms have appeared, and also occasionally in septicæmic types without meningitis. For a considerable time it has been known that contacts with cases of cerebro-spinal fever often harbour the meningococcus in the naso-pharynx, that is, are "carriers," and during the war this subject was extensively investigated, the examination of contacts becoming a routine procedure. The proportion of "positives" among contacts varies, 20 per cent. or higher. Non-contacts have been examined during epidemics, and among them also a considerable proportion, though not so great as among contacts, have been found to be carriers. Among troops during the war the normal carrier rate was about 2 to 4 per cent. In a report by the Ministry of Health (1930) it was clearly shown that a certain proportion of meningococcal carriers may be found in the normal population: in the series of observations dealt with 2·44 per cent. of the persons examined were carrying the organism. In troops it was noted that just prior to an epidemic of cerebro-spinal meningitis the carrier rate increased and after it reached 20 per cent., or over, cases of the active disease appeared. It was also observed that in overcrowded quarters the carrier rate was high, whereas the spacing out of the beds effected a marked decrease in the percentage of carriers. Thus overcrowding is one of the most active factors in promoting the spread of the disease and it has been generally accepted that the occurrence of an epidemic is conditioned by the prevalence of carriers. Dudley and Brennan have recently shown, however, that a high carrier rate is not necessarily accompanied by cases of meningitis; they have recorded in one instance a carrier rate of 50 per cent. over a period of a year without associated meningitis. In some carriers the organism occurs sparsely among other organisms, but in others in fairly large proportion, and occasionally in almost pure culture. In the great majority of carriers the organism can be found for only a comparatively short time—a few days even, or a week or two—but in a small proportion it persists for months, these being

"chronic" carriers. Unfortunately, we have at present no ready means of estimating the relative virulence of meningococci obtained from the naso-pharynx and from elsewhere. With regard to the epidemiology certain facts are outstanding. One is that direct infection of a healthy individual from a patient suffering from the disease is comparatively uncommon, though it sometimes occurs; the other is that it is rare for a known carrier to develop the disease. On the other hand, there is substantial evidence of persons being infected from carriers. The fact mentioned would seem to show that the organism is spread widely from individual to individual, in most cases without result, but that when the organism reaches a susceptible individual the disease may rapidly develop; in this connection the high susceptibility of young children must be remembered. No doubt the number of the organisms in the naso-pharynx of the carrier is a factor of importance. It has been stated by some observers that the presence of the meningococcus leads to, or is associated with, naso-pharyngeal catarrh, and that this often precedes meningeal infection. In fact, the primary lesion may be a naso-pharyngitis. On the other hand, the organism may abound in the naso-pharynx, without the presence of catarrh or any abnormality. Manifestly the act of coughing will aid in its diffusion when it is present.

Apart from the epidemic form of the disease, cases of a sporadic nature also occur, in which the lesions are of the same nature, and in which the meningococcus is present. The facts stated would indicate that the origin and spread of the disease in the epidemic form depend on certain unknown conditions which produce an increased virulence of the organism.

Identification of the Meningococcus: Serological Types.—In the case of meningitis, this usually presents no difficulty, as the finding of a Gram-negative diplococcus in the cerebro-spinal fluid is practically conclusive. In the case of the naso-pharynx, however, the matter is quite different. Means must be taken to distinguish the organism from others resembling it, which occur in this situation Originally, the following points taken together were usually accepted as justifying a positive diagnosis: *conformity in the microscopic characters and in the appearance of the colonies with those of the meningococcus, ready emulsification in saline, absence of growth on agar at room temperature, fermentation of glucose and maltose, and non-fermentation of saccharose.* Attempts have been made to obtain identification by means of agglutination. Dopter was the first to show that strains obtained from the naso-pharynx, in other respects conforming to the meningococcus, might not be agglutinated by the serum of an animal immunised against a known strain of this organism; he applied the term *parameningococcus* to such strains.

During the war Gordon found that a serum prepared by injecting any one strain did not agglutinate all the strains isolated from the cerebro-spinal fluid and naso-pharynx. Proceeding further, he arrived finally at the recognition of four " types " (I.–IV.), according to agglutinating tests, cross agglutination between them being little marked. Of these, Types I. and II. were the commonest, the latter being rather the more frequent. All the strains separated from cases of meningitis were found to be agglutinated by one of the four sera. Gordon's results in military cases proved of much practical value, but they have been criticised by Griffith and Scott and by Fildes, who point out that not all meningococci are agglutinated by one of Gordon's monovalent type sera, and that accordingly some meningococci in the naso-pharynx may escape detection by this method of testing. Fildes also states that while the rabbit tends to produce a monovalent serum on being inoculated with a strain of meningococcus, the horse when similarly inoculated may produce a serum which is polyvalent. At present it appears hardly justifiable to conclude that an organism may not be the meningococcus merely because it is not agglutinated by certain sera, provided that it conforms in other respects.

Griffith has classified meningococci serologically on a different basis from that of Gordon. He divided them into two groups (I. and II.) by direct agglutination tests. Group I. embraced Gordon's Types I. and III., and Group II. included Types II. and IV. According to Griffith, Group I. is more complex in antigenic structure than Group II. Griffith found also that strains isolated from the pharynx could be similarly classified, but only a minority belonged to Group I. He suggested a relationship between virulence and antigenic complexity, and Andrewes has also concluded that increase of virulence may be associated with greater complexity in antigenic constitution. Single colonies from a particular strain may vary in antigenic structure as shown by Macgraith who points out that Types I. and III. are not fixed and that there is an overlap between them. Rough variants may occur. Branham in the United States who also found that Types I. and III. are difficult to separate, originally noted overlapping between II. and IV., though in later years these types became more distinct. Segal has reported the occurrence of a " Type V." meningococcus.

Chemical studies of the antigenic constituents of these organisms have revealed polysaccharide and protein factors which the meningococcus shares with other Gram-negative diplococci, and more specific carbohydrate and protein substances have also been demonstrated (Zozaya and Wood, Rake and Scherp), but these studies are not sufficiently complete to provide a definite antigenic analysis of the organism on a chemical basis.

An interesting method of eliciting precipitation reactions with meningococci has been devised by Petrie who grows the organism on solid medium in which an antiserum is incorporated. The occurrence of a halo round the colony denotes the production of precipitate, the reacting constituent of the organism having diffused from the growth into the surrounding medium.

Preparation of Agglutinating Sera.—Hine has devised the following method in the case of meningococci. A rabbit receives on one day three intravenous injections of 500 millions of dead meningococci, with an interval of an hour between the injections ; six days after-

wards it receives a single dose of 3000 millions. Eight days later the serum has usually a titre of over 1 : 800. Young rabbits of about a kilogram in weight give the best results. For testing, four dilutions are used, namely: 1 : 50, 1 : 100, 1 : 200, 1 : 400. Emulsions of known type organisms are used as controls at the same time. After the mixtures are made they are placed at 55° C. for twenty-four hours, and the results are then read.

Precipitin Reaction with the Cerebro-spinal Fluid in Cases of Meningitis.—Rake has identified the type of meningococcus responsible for infection by placing 0·1 c.c. of each type of antiserum in a series of small tubes and then placing on the surface 0·1 c.c. of the spinal fluid which has been previously cleared by centrifuging. The tubes are kept in a water bath at 37° C. for one hour, then inspected and again kept at 37° C. for one hour and thereafter in the ice-chest over night. In the majority of cases of meningococcus infection a ring of opacity develops in the tube containing the corresponding antiserum.

Serum Reactions in Cases of Meningitis.—An agglutination reaction towards the meningococcus is given by the serum of patients suffering from the disease, when life is prolonged for a sufficient length of time. It usually appears about the fourth day, when the serum may give a positive reaction in a dilution of 1 : 50; at a later stage it has been observed in so great a dilution as 1 : 1000. Specific opsonins may appear in the blood about the same time, and though they are not always proportional in amount to the agglutinins, the two classes of substances have more or less the same significance, and may occasionally be of use in diagnosis when lumbar puncture fails to give positive results. Although their presence in large amount may be said to indicate a marked reaction, they do not supply information of much value in relation to prognosis. Immune-bodies, as shown by bactericidal and fixation of complement tests (pp. 223, 226), may also be developed in considerable amount in the course of the disease. On the other hand, carriers appear not to develop antibodies in their serum.

Therapeutic Antisera.—These have been introduced by various workers, and the type of serum which has been most extensively used is that originally prepared by Flexner and Jobling. The serum was obtained from the horse by repeated injections in increasing doses of dead cultures, followed by injections of culture autolysate and of living cultures, these two latter being best administered by the subcutaneous method. Several strains of meningococci were mixed together for purposes of injection, and the immunisation was continued over a period of several months. For treatment of the disease anti-meningococcal serum is injected under the spinal dura, 30 c.c. being generally used for an injection in an adult, this being repeated on subsequent days, or even twice daily. Some of the spinal fluid is removed, and then the serum is injected, undue pressure being avoided. Intravenous injections of serum should be combined with the intrathecal administration. This serum has

been used on a large scale in various parts of the world, and there is general agreement as to its favourable effects—thus it is claimed that the mortality of the disease, which is generally 70 to 80 per cent., has been reduced to about 30 per cent. or even less, and the tendency to the occurrence of chronic lesions has also been markedly diminished. The later results, however, especially in the treatment of sporadic cases of meningococcal meningitis in children have been unsatisfactory. The action of such antisera cannot as yet be fully explained. They contain opsonins, agglutinins, immune-bodies which fix complement, and possibly also anti-endotoxins. After the injection the numbers of meningococci are apparently reduced, probably as a result of increased phagocytosis; there can scarcely be any direct bactericidal action owing to the absence of complement. During the war monovalent sera against each of the four types of meningococcus and also a polyvalent serum were prepared for military cases by Gordon and his co-workers. The standardisation of such antisera has proved a matter of some difficulty; at first the fixation of complement method was used, but later the opsonic index was regarded with more favour as a criterion of the potency of the serum. Gordon has pointed out the importance of estimating the antitoxic action, and has described a method for this purpose. The agglutination reaction and a protection test in animals have also been advocated. It is doubtful if any of these methods provides a reliable means of regulating the therapeutic potency of antimeningococcal sera and in all probability different antisera and different batches of serum prepared by the same method vary considerably in this respect.

Mackenzie and Martin treated cases by the intraspinal injection of the fresh serum of patients suffering from the disease or who had recovered from it, such serum being in many cases rich in immune-bodies for the meningococcus, and possessing a greatly increased bactericidal action as compared with normal serum.

Allied Diplococci.—In the naso-pharynx there occur other Gram-negative diplococci which morphologically have a close resemblance to the meningococcus. Many of these are stated to be chromogenic, producing yellow or yellowish-green pigments, e.g. *M. flavus* (*Neisseria flava*), and can thus be distinguished. Three varieties of this organism have been described: two of them grow on ordinary media at room temperature but have fermentative reactions similar to the meningococcus; the other type does not flourish at low temperature but ferments saccha-

rose (cf. meningococcus). *Diplococcus* or *Micrococcus catarrhalis* (*Neisseria catarrhalis*) has also a close resemblance to the meningococcus. In addition to occurring in health this organism has been found in large numbers in catarrhal conditions of the pharynx and respiratory passages. Its microscopic appearances are practically similar to those described above, and it also occurs within leucocytes. Its colonies on serum agar, though on the whole tending to be rather more opaque, closely resemble those of the meningococcus, but are rather tough in consistence and not so readily emulsifiable. The organism usually grows at 20° C., and it has none of the fermentative properties described above as belonging to the meningococcus (p. 352). The *Diplococcus pharyngis siccus* (*Neisseria sicca*) grows at room temperature, and its colonies are very tough and adhere to the surface of the medium; it can thus readily be distinguished from the meningococcus. It has marked fermentative properties, acting on glucose, maltose, saccharose, and lævulose. This organism is probably a "rough" variant of another type of commensal diplococcus of the throat. The *Diplococcus mucosus* has colonies of slimy consistence; it grows at room temperature, and it forms capsules, which can be demonstrated by the method of Hiss. The points of difference between the meningococcus and the gonococcus are given on p. 367. Other species of Gram-negative diplococci have been distinguished, but have no pathogenic importance so far as is known. A Gram-positive diplococcus called the *Diplococcus crassus* is also of common occurrence; it is rather larger than the meningococcus, and, especially in subcultures, may tend to assume staphylococcal forms. Its Gram staining is variable. It grows on ordinary media and at room temperature. The colonies are very small and not unlike those of a streptococcus. Glucose, maltose, lactose, and saccharose are fermented.

Branham, in a recent small outbreak of meningitis in America, isolated from the cerebro-spinal fluid in a proportion of cases a hitherto undescribed meningococcus-like organism (*Neisseria flavescens*) which, growing on blood agar, produced golden-yellow pigment; it fermented none of the usual carbohydrates. Agglutination tests with specific antisera indicated that the strains were serologically identical; the organisms were not agglutinated by antisera to any of the four types of meningococci.

Meningitis due to other Organisms.—Meningitis may also be produced by almost any of the pyogenic organisms. A considerable number of cases, especially in children, are due to the *pneumococcus*. In many instances where no other lesions are

present the extension is by the Eustachian tube to the middle ear. In other cases the path of infection is from some other lesion by means of the blood stream. This organism also infects the meninges not infrequently in lobar pneumonia, and in some cases with cerebral symptoms we have found it present where there was merely a condition of congestion. Occasionally epidemics of meningitis have been due to the pneumococcus. Sporadic cases are also met with, and this organism comes next in order of frequency to the meningococcus as the cause of primary meningitis. The *pneumobacillus* also has been found in a few cases. Meningitis is not infrequently produced by *streptococci*, especially when middle-ear disease is present, less frequently by one of the *staphylococci* ; occasionally more than one organism may be concerned. In meningitis following influenza *Bacillus influenzæ* has been found in a few instances, but sometimes the pneumococcus is the causal agent. Sporadic cases of meningitis occur associated with organisms which resemble the influenza bacillus morphologically and also in presenting hæmophilic culture reactions, but which possess marked pathogenic properties for rabbits and guinea-pigs. Both in the cerebro-spinal fluid and in cultures, these bacilli frequently show a tendency to produce long filamentous forms and also may show a beading of the protoplasm (*vide* p. 580). The cases from which such bacilli have been isolated have chiefly occurred in children, are extremely fatal, and may follow on an otitis media, from which condition similar organisms have been isolated. Sometimes the meningitis is part of a septicæmia or pyæmic process—in the latter the joints are often affected. The biological relationships of the organisms associated with such conditions will be dealt with later (p. 585). They certainly tend to be more widely distributed in the body of the infected individual than is the case in influenza. An invasion of the meninges by *B. anthracis* occurs, but is a rare condition ; it is attended by diffuse hæmorrhage in the sub-arachnoid space. In tuberculous meningitis the *tubercle bacillus*, of course, is present, especially in the nodules along the sheaths of the vessels.

Various other pathogenic organisms have on occasions been reported as producing meningitis, *e.g.*, gonococcus, *B. typhosus*, *B. abortus*, *Actinomyces* ; meningitis may be a feature of infection by the poliomyelitis virus (p. 756) and recently cases of meningitis have been described which are due to another filterable virus (*vide* lymphocytic choriomeningitis, p. 762).

In conclusion, it may be stated that *mixed infections* may

occur in meningitis. Thus the pneumococcus has been found associated with the tubercle bacillus and also with the meningococcus, sometimes appearing as an additional infection to the latter.

Methods of Examination.—During life these involve the microscopic investigation of the centrifuged cerebro-spinal fluid and making cultures therefrom (p. 167). For the former, smears stained by methylene-blue and by Gram's method make the recognition of the meningococcus relatively easy, and the presence of Gram-negative cocci, especially within cells, is practically diagnostic of a case of cerebro-spinal fever. Tubes or plates of Huntoon's agar, serum agar, boiled-blood agar (pp. 60, 69) may then be inoculated; this should be done without delay, as the organisms tend to die in the course of a few hours when the cerebro-spinal fluid is kept at room temperature. The difficult cases are those where no bacteria can be found microscopically in the spinal fluid. Here the character of the exudate may give help. A predominance of polymorphonuclear cells is usually manifest in meningococcal, pneumococcal, and *B. influenzae* infections, whereas in tuberculous meningitis the exudate is, as a rule, chiefly lymphocytic, though polymorphs, often degenerated, also occur. In such circumstances, besides other media, blood agar should be inoculated in case the pneumococcus or *Bacillus influenzæ* is the causal organism. Also several c.c. of the fluid mixed with an equal volume of 1 per cent. glucose broth should be incubated at 37° C., as by this means meningococci can sometimes be recovered when no growth results from the sediment. To speak generally, if with a polymorphonuclear exudate no growth occurs in the media mentioned, the case is most likely to be due to the meningococcus. The isolation of the organism from the naso-pharynx will give confirmatory, though of course not conclusive, evidence. It must be kept in view, however, that in meningitis high up, produced by any of the organisms mentioned, polymorphonuclear leucocytes may be present in the fluid obtained by lumbar puncture before the organisms themselves appear. It should also be noted that a polymorph exudate may be observed in the spinal fluid in cases of poliomyelitis (p. 756). In tuberculous cases it is sometimes impossible to demonstrate the bacilli microscopically in the exudate, though on careful search they may usually be found.

For method of examination of the naso-pharynx, *vide* p. 168. The recognition of meningococci in cultures from the nasopharynx of suspected carriers and their differentiation from

other Gram-negative diplococci have already been discussed (pp. 356, 359).

The Gonococcus and Gonorrhœa

Introductory.—The micrococcus now known to be the cause of gonorrhœa, and called the *Gonococcus*, was first described by Neisser, who in 1879 gave an account of its microscopical characters as seen in the pus of gonorrhœal affections, both of the urethra and of the conjunctiva. He considered that this organism was peculiar to the disease, and that its characters were distinctive. Later it was successfully isolated and cultivated on solidified human serum by Bumm and others. Its characters have since been minutely studied, and by inoculation of cultures in the human subject its causal relationship to the disease has been conclusively established.

The Gonococcus (*Neisseria gonorrhœæ*).—*Microscopical Characters*.—The organism of gonorrhœa is a micrococcus (about 0·8 to 1 μ in diameter) which usually is seen in diplococcal form, the adjacent margins of the two cocci being flattened, or even slightly concave, so that between them there is a small oval unstained interval. An appearance is thus presented which has been compared to that of two beans placed side by side (Fig. 56). When division takes place in the two members of a diplococcus, a tetrad is formed, which, however, soon separates into two sets of diplococci—that is to say, arrangement as diplococci is much commoner than as tetrads. Cocci in process of degeneration are seen as spherical elements of varying size, some being considerably swollen and staining faintly.

These organisms are found in large numbers in the pus of acute gonorrhœa, both in the male and female, and for the most part are contained within the leucocytes. In the earliest stage, when the secretion is glairy, a considerable number are lying free, or are adhering to the surface of desquamated epithelial cells, but when it becomes purulent the large proportion within leucocytes is a very striking feature. In the leucocytes they lie within the protoplasm, especially superficially, and are often so numerous that the leucocytes appear to be filled with them, and their nuclei are obscured. It has been observed that the marked and characteristic phagocytosis of the organism only occurs in the purulent exudate and not in the inflamed tissue. As the disease becomes more chronic, the gonococci gradually become fewer, though even in long-standing cases they may still be

found in considerable numbers. They are also present in the purulent secretion of gonorrhœal conjunctivitis, in various parts of the female genital organs when these are the seat of gonorrhœal infection, and they have been found in some cases in the secondary infections of the joints, as will be described below.

Staining.—The gonococcus stains readily and deeply with a watery solution of any of the basic aniline dyes—methylene-blue, fuchsin, etc. It is, however, easily decolorised, and is Gram-negative—an important point in microscopical diagnosis.

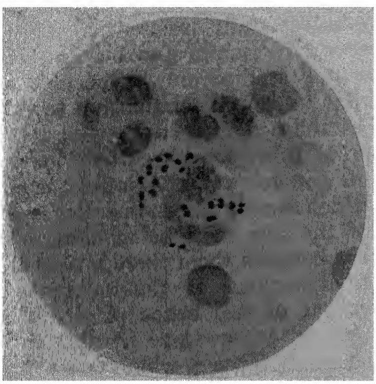


FIG. 56.—Portion of film of gonorrhœal pus, showing the characteristic arrangement of the gonococci within leucocytes. See also Plate I., Fig. 5. Stained by Gram's method and dilute carbol-fuchsin. ×1000.

Cultivation. — This is attended with some difficulty, as the conditions of growth are somewhat restricted. Blood or serum, incorporated in the culture medium, is necessary, and a variety of media have been advocated by different workers for the cultivation of the organism. The most suitable for routine culture are blood agar, boiled-blood agar, and serum media[1] (p. 69). The optimum pH is from 7·3 to 7·6 (Tulloch). It is advisable to inoculate the media within half an hour after obtaining the material from the body, and to place the tubes or plates at once in the incubator. Growth takes place best at 35° to 36° C. (Jenkins), and ceases altogether at 25° C. The organism requires abundant moisture for its growth. Torrey and Buckell have advocated the use of a semi-solid agar in place of the ordinary solid medium. If tubed medium is employed, there should be sufficient condensation fluid present. A suitably moist atmosphere for the growth of the gonococcus can be obtained by inoculating tubes or plates in an airtight jar containing a pad of wet glass-wool (Tulloch). Cultures are obtained by taking some pus on the loop of the platinum wire and

[1] Certain special media have been used with much success, *e.g.* by Tulloch (*Journ. Path. and Bact.*, 1922, xxv. 346).

inoculating plates of medium by successive strokes. The colonies are usually visible within forty-eight hours, and often within twenty-four hours; it is important, however, to note that sometimes growth may not appear till the fourth day. Different strains seem to vary in their ability to grow on artificial media in primary culture. According to McLeod and his co-workers, the growth of many strains is promoted by incubation in air containing 8 per cent. carbon dioxide. The colonies are small, semi-transparent, rounded discs, and vary somewhat in size. They tend to remain more or less separate. Later, the margin may become undulated and the centre more opaque; radial and concentric markings may be present (Fig. 57). The first cultures die out somewhat quickly, but in subcultures,

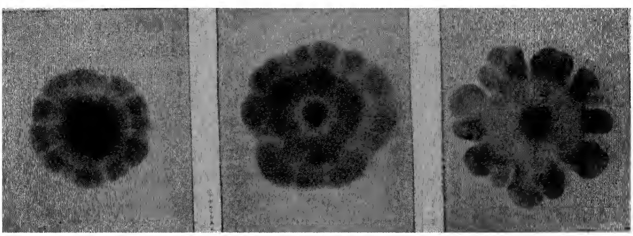


FIG. 57.—Colonies of gonococcus on serum agar, (*a*) three days' growth; (*b*) and (*c*) five days' growth. ×9.
From photographs by the late Prof. W. B. M. Martin.

kept at 37° C., the organism remains alive for a considerable time, sometimes three weeks. After about a week, more active foci of growth may appear in some of the colonies in the form of papillæ. Atkin has drawn attention to two types of colonies which apparently represent different biological types of the organism: (I.) large, irregular, thin colonies with papillæ; (II.) smaller, rounder, thicker, and denser colonies without papillæ. He claims that the papillæ of Type I. are more viable than their substrate, and that Type II. colonies are derived from these papillæ of Type I.

McLeod and his co-workers have found that the recognition of gonococcal colonies is aided by the use of the direct oxidase reaction: cultures are made on heated-blood agar in plates and after two days' incubation are covered with a 1 per cent. solution of tetramethyl-p-phenylenediamine hydrochloride which is immediately run off; medium-sized convex and translucent colonies, which rapidly turn purple, are accepted for

diagnostic purposes as gonococci if they consist of Gram-negative diplococci.

The special requirements for the growth of the gonococcus have been studied by various workers. Amino-acids are essential, but McLeod and co-workers have shown that while certain amino-acids favour growth, others may exert an inhibitory action, depending on their concentration and the presence of blood. Thus alanine-d and taurine, while inhibitory in certain concentrations, favour growth if 0·5 per cent. of blood is added to the mèdium. Whole-blood acts better than serum in this respect, and blood heated at 55° C. better than fresh blood. An important function, therefore, of blood added to culture medium is the neutralisation of any growth-inhibitory properties of particular amino-acids present in the commercial peptone used. Some workers have stressed the value of adding 1 per cent. disodium hydrogen phosphate to the medium; this probably acts in virtue of its buffering property. Thomson has recommended the addition of 2 to 3 per cent. glucose.

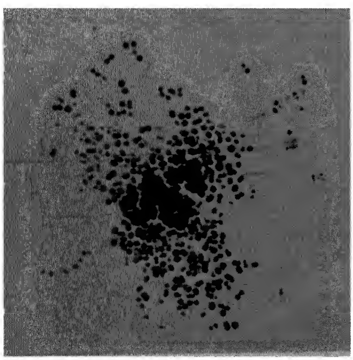


FIG. 58.—Gonococci, from a pure culture on blood agar of twenty-four hours' growth. Some cocci already are beginning to show the swollen appearance common in older cultures. Stained with carbol-thionin. ×1000.

In the early stage of the disease the organism is present in the male urethra in practically pure condition, and if the meatus of the urethra be sterilised by washing with a weak solution of corrosive sublimate and then with absolute alcohol, and the material for inoculation be expressed from the deeper part of the urethra, cultures may often be obtained which are practically pure from the first. In culture, the organisms have similar microscopic characters to those described (Fig. 58), but show a remarkable tendency to undergo degeneration, becoming swollen and of various sizes, and staining very irregularly. Degenerated forms are seen even on the second day, while in a culture four or five days old comparatively few normal cocci may be found. The less suitable the medium the more rapidly does degeneration take place. When mixed with other organisms the gonococcus may be separated in the usual way by plate cultures on the media mentioned.

Comparison with Meningococcus.—The morphological and cultural characters of the gonococcus and meningococcus are in many

respects closely similar; the following points are of importance in distinguishing them biologically. The conditions of growth of the gonococcus are more restricted than those of the meningococcus. The gonococcus does not grow on the ordinary agar media, whereas the meningococcus may grow slightly after repeated subculture. The colonies of the latter are generally more opaque and have more regular margins than those of the gonococcus. In fermentative reactions glucose is the only sugar usually employed which is fermented by the gonococcus, whereas the meningococcus ferments maltose also. (For fermentation tests in the case of the gonococcus, solid media should be used, sloped agar containing 5 per cent. sterile unheated serum, with litmus and the particular sugar added, being specially suitable.) It has been stated that the meningococcus differs from the gonococcus in its power to produce hæmolysis when growing on 5 per cent. horse-blood agar (Joachimovits).

Serological Types of the Gonococcus.—A study of the immunity reactions of the gonococcus with antisera has elicited the fact that this species of organism is serologically heterogeneous. The agglutination and agglutinin-absorption reactions have been mostly studied, and the general result of different workers has been that gonococcus strains can be classified into a number of serologically distinct types. Torrey and Buckell find that gonococcus strains cannot be classified into such well-defined serological types as in the case of the pneumococcus (*q.v.*), and that certain strains possess generalised antigenic characters common to the species as a whole. They have also observed variations in individual strains at different times as regards their antigenic properties. According to Tulloch 72 per cent. of cases of acute and subacute gonorrhœa in the male are caused by one fairly well-defined serological type.

The antigenic constituents of the gonococcus have been studied recently by chemical methods and precipitin reactions as in similar studies of the pneumococcus (*q.v.*). Nucleo-protein and polysaccharide factors have been separated and it would appear that certain constituents are common to this organism and to the meningococcus and pneumococcus Type III. (Miller and Boor).

Relations to the Disease.—The gonococcus is invariably present in the urethral discharge in gonorrhœa, and also in other parts of the genital tract when these are the seat of gonorrhœal infection. Its presence in these different positions has been demonstrated not only by microscopical examination but also by culture. From the description of the conditions of growth in culture it will be seen that life outside the body in natural conditions is practically impossible—a statement which corresponds with the clinical fact that the disease is always transmitted directly by contagion. Inoculation of pure cultures in the urethra of lower animals, and even of apes, is followed by no effect, but a similar statement can be made with regard to inoculation of gonorrhœal pus itself. In fact, hitherto it has been found impossible to reproduce the disease by any means in the lower animals. On a considerable number of occasions

inoculations of pure cultures have been made in the human urethra, both in the case of the male and female, and the disease, with all its characteristic symptoms, has resulted. (Such experiments have been performed independently by Bumm, Steinschneider, Wertheim, and others.) The causal relationship of the organism to the disease has therefore been completely established.

Intraperitoneal injections of pure cultures of the gonococcus in white mice produce a localised peritonitis with a small amount of suppuration, the organisms being found in large numbers in the leucocytes (Wertheim). They also penetrate the peritoneal lining and are found in the sub-endothelial connective tissue, but they appear to have little power of proliferation, they soon disappear, and the inflammatory condition does not spread. Injection of pure cultures into the joints of rabbits, dogs, and guinea-pigs causes an acute inflammation, which, however, soon subsides, whilst the gonococci rapidly die out; a practically similar result is obtained when dead cultures are used. These experiments show that while the organism, when present in large numbers, can produce a certain amount of inflammatory change in these animals, it has little or no power of multiplying and spreading in their tissues.

Toxin of the Gonococcus.—De Christmas cultivated the gonococcus in a mixture of 1 part of ascitic fluid and 3 parts of broth, and found that the fluid after twelve days' growth had toxic properties. At this period all the organisms were dead, and the fluid constituted the "toxin." The toxic substances were precipitated along with the proteins by alcohol, and the precipitate after being desiccated possessed the toxic action. In young rabbits injection of the toxin produced suppuration; this was well seen in the anterior chamber of the eye, where hypopyon resulted. The most interesting point, however, was with regard to its action on mucous surfaces; for, while in the case of animals it produced no effect, its introduction into the human urethra caused acute catarrh, attended with purulent discharge. He found that no tolerance to the toxin resulted after five successive injections at intervals. In a later publication he pointed out that the toxin on intra-cerebral injection had marked effects; he also claimed to have produced an antitoxin. The production of a soluble toxin as described by De Christmas has not been confirmed by subsequent workers, though recently Clark, Ferry, and Steele have stated that a soluble toxin can be demonstrated in gonococcal cultures and that this product is antitoxinogenic.

Distribution in the Tissues.—The gonococcus having been thus shown to be the direct cause of the disease, some additional facts may be given regarding its presence both in the primary and secondary lesions. In the human urethra the gonococci penetrate the mucous membrane, passing chiefly between the epithelial cells, causing a loosening and desquamation of many of the latter and inflammatory reaction in the tissues below.

attended with great increase of secretion. There occurs also a gradually increasing emigration of leucocytes, which take up a large number of the organisms. The organisms also penetrate the subjacent connective tissue and are especially found, associated with extensive leucocytic emigration, around the lacunæ. Even, however, when the gonococci have disappeared from the urethral discharge, they may still be present in the deeper part of the mucous membrane of the urethra, and also in the prostate, and may thus be capable of producing infection. The prostatic secretion may be examined by making pressure on the prostate from the rectum ("prostatic massage") when the patient has almost emptied his bladder, the secretion being afterwards discharged along with the remaining urine. In acute gonorrhœa there is often a considerable degree of inflammatory affection of the prostate and vesiculæ seminales, but it is difficult to say whether these conditions are always due to the presence of gonococci in the affected parts. A similar statement applies to the occurrence of orchitis and also of cystitis in the early stage of gonorrhœa. Gonococci have, however, been obtained in pure culture from peri-urethral abscesses and from epididymitis: it is likely that the latter condition, when occurring in gonorrhœa, is usually due to the actual presence of gonococci. During the more chronic stages other organisms may appear in the urethra, aid in maintaining the irritation, and may produce some of the secondary results. The pyogenic cocci, *B. coli*, diphtheroid bacilli, etc., are often present, and may extend along the urethra to the bladder and set up cystitis, though in this they may be aided by the passage of a catheter. It may be mentioned here that Wertheim cultivated the gonococcus from a case of chronic gonorrhœa of two years' standing, and by inoculation in the human subject proved it to be still virulent.

In the disease in the female, gonococci are almost invariably present in the urethra, the situation affected next in frequency being the cervix uteri. They do not appear to infect the lining epithelium of the vagina of the adult unless some other abnormal condition be present, but they do so in the gonorrhœal vulvovaginitis of young subjects. They have also been found in suppurations in connection with Bartholin's glands, and sometimes produce an inflammatory condition of the mucous membrane of the body of the uterus. They may also pass along the Fallopian tubes and produce inflammation of the mucous membrane there. From the pus in cases of pyosalpinx they have been cultivated in a considerable number of cases. Accord-

24

ing to the results of various observers they have been found in one out of four or five cases of this condition, usually unassociated with other organisms. Further, in a large proportion of the cases in which the gonococcus has not been found, no organisms of any kind have been obtained from the pus, and in these cases the gonococci may have been once present and have subsequently died out. Lastly, they may pass to the peritoneum and produce peritonitis, which is usually of a local character.

In *gonorrhœal conjunctivitis* the mode in which the gonococci spread through the epithelium to the subjacent connective tissue is similar to what obtains in the case of the urethra. Their relation to the leucocytes in the purulent secretion is also the same. Microscopic examination of the secretion alone in acute cases often gives positive evidence, and pure cultures may be readily obtained. As the condition becomes more chronic, gonococci are less numerous and a greater proportion of other organisms may be present. An infection of the conjunctiva in new-born children, *ophthalmia neonatorum*, which is often of very severe nature, is also due to the gonococcus.

Relations to Joint-Affections, etc.—The relations of the gonococcus to the sequelæ of gonorrhœa form a subject of great interest and importance. The following statements may be made with regard to them : First, in a certain number of cases of arthritis following gonorrhœa pure cultures of the gonococcus may be obtained. A similar statement applies to inflammation of the sheaths of tendons following gonorrhœa. Secondly, in a considerable proportion of cases no organisms have been found. It is, however, possible that in many of these the gonococci may have been present in the synovial membrane, as it has been observed that they may be much more numerous in that situation than in the fluid. Thirdly, in some cases, especially in those associated with extensive suppuration, occasionally of a pyæmic nature, various pyogenic cocci have been found to be present. In the instances in which the gonococcus has been found in the joints, the fluid present has usually been described as being of a whitish-yellow tint, somewhat turbid, and containing shreds of fibrin-like material, though sometimes purulent in appearance. In one case Bordoni-Uffreduzzi cultivated the gonococcus from a joint-affection, and afterwards produced gonorrhœa in the human subject by inoculating with the cultures obtained. In another case, in which pleurisy was present along with arthritis, the gonococcus was cultivated from the fluid in the pleural cavity. It seems possible that gonorrhæal arthritis may also occur as a manifestation of allergy to the products of the

organism, as in streptococcal infection (*vide* p. 307). The existence of a *gonorrhœal endocarditis* has been established. Cases apparently of this nature occurring in the course of gonorrhœa had been previously described, but the complete bacteriological test has been satisfied in several instances. In one case Lenhartz produced gonorrhœa in the human subject by inoculation with the organisms obtained from the vegetations. A true *gonorrhœal septicæmia* has also been demonstrated, cultures of the gonococcus having been obtained from the blood during life on more than one occasion (Thayer and Blumer, Thayer and Lazear, Ahmann). Such occurrences are, however, rare.

Immunity and Serum Diagnosis.—Immunity following recovery from the infection is usually transient. Specific antibodies have been demonstrated in the serum of persons infected with the gonococcus, *e.g.* agglutinin, and complement-fixing immune-body; and the complement-fixation test has now been extensively applied in the diagnosis of gonorrhœal infections in which the organism cannot be demonstrated by direct methods. The general principles and technique of the method are those described in Chapter IV. For the test a polyvalent antigen must be used, consisting of a number of strains sufficient to represent the different serological races of the gonococcus.

Price recommends an antigen prepared as follows: the organism is grown on hydrocele agar in a Roux flask and washed off with 100 c.c. saline solution; 1 c.c. of normal sodium hydrate is added and the suspension is placed at 37° C. for two hours; this brings about dissolution of the organisms and the material is then filtered through lint; 1·5 c.c. normal hydrochloric acid is added to the filtrate and the fluid is placed again at 37° C.; a flocculent precipitate forms within twenty minutes and is separated by centrifuging and suspended in 4 c.c. saline solution; the suspension is then adjusted to pH 7·5 by the addition of decinormal sodium hydrate when, after shaking, the precipitate undergoes solution; 1 c.c. of 1 per cent. formol-saline is added and the fluid after filtration is used as the antigen diluted 1 in 30 for the actual test.

According to Price, in the first week of a gonorrhœal infection 27 per cent. of cases yield a positive reaction with this antigen and the percentage of reactors increases with the duration of the illness, 100 per cent. giving the reaction after five weeks. Repeated negative reactions after treatment have been regarded as indicative of cure.

Allergy.—A cutaneous allergic reaction can be elicited in certain cases by the intradermal injection of killed organisms, *e.g.* gonococcus vaccine. It is negative in the early stage of the infection, and though frequently positive in the later stages is not uniformly so. The specificity of the reaction is not complete.

Vaccines.—Both gonorrhœa itself and the secondary infections have been treated by means of vaccines, but the results reported vary greatly. On the whole, most success has been obtained in the case of joint infections and allied conditions, though even here

reports are contradictory. The initial dose employed has been usually about five million cocci, but care is necessary in starting the treatment, especially in the case of acute gonorrhœa.

Serum Therapy.—Specific antisera have been applied in the treatment of gonorrhœa, but the results have not been such as to encourage their general use.

Methods of Diagnosis.—For microscopic examination, dried films of the suspected pus, etc., may be stained by any of the simple solutions of the basic aniline stains. Methylene-blue or thionin may be used. Staining for one minute is sufficient. It is also necessary to stain by Gram's method, and it is advisable to put alongside the pus film a small quantity of culture of staphylococcus as a "control spot" in staining by this method; the control should stain deeply violet, while the gonococci are coloured only with the counter-stain. Regarding the value of microscopic examination alone, we may say that the presence in a urethral discharge of a large number of diplococci having the characters, position, and staining reactions described above, is practically conclusive that the case is one of gonorrhœa. There is no other condition in which this sum-total of microscopical characters is present. A similar statement applies to conjunctivitis. Even in chronic cases the typical picture is often well maintained, and microscopic examination alone may give a definitely positive result. When other organisms are present, and especially when the gonococci are few in number, it is difficult, and in some cases impossible, to give a definite opinion, as a few gonococci mixed with other organisms cannot be recognised with certainty. This is often the condition in chronic gonorrhœa in the female. In the case of the female a drop of secretion should be taken on a platinum loop from the urethra or, with the aid of a speculum, from the cervix uteri, the adjacent parts being cleansed as far as possible by swabbing with sterile cotton wool. Microscopic examination, therefore, though often giving positive results, will sometimes be inconclusive. In such cases cultures on suitable media (*vide supra*) should be made if possible, though in a mixed infection it may be difficult to isolate and identify the organism. The direct oxidase reaction, referred to above, may facilitate the recognition of colonies of the gonococcus. As regards lesions in other parts of the body, microscopic examination alone is quite insufficient; it is impossible, for example, to distinguish by this means the gonococcus from other Gram-negative diplococci. Cultures alone supply the test, and the points above detailed are to be attended to.

The complement-fixation test (*vide supra*) may be applied in suspected cases in which gonococci cannot be demonstrated by direct methods.

CHAPTER IX

THE DIPHTHERIA BACILLUS AND ALLIED ORGANISMS

THERE is no better example of the valuable contributions of bacteriology to scientific medicine than that afforded in the case of diphtheria. Not only has research supplied a means of distinguishing true diphtheria from conditions which resemble it, but the study of the toxins of the bacillus has explained the manner by which the pathological changes and characteristic symptoms of the disease are brought about, and has led to the discovery of specific means of prophylaxis and treatment.

Historical.—Bretonneau, from clinical and pathological observations, distinguished diphtheria as a specific disease; but the proof of this was effected by the discovery of the specific causal organism. The first account of the bacillus now known to be the cause of diphtheria was given by Klebs in 1883, who described its characters in the false membrane, but made no cultivations. It was first cultivated by Löffler from a number of cases of diphtheria, his observations being published in 1884, and to him we owe the first account of its characters in cultures and some of its pathogenic effects on animals. The organism is for these reasons known as the Klebs-Löffler bacillus. By experimental inoculation with the cultures obtained, Löffler was able to produce false membrane on damaged mucous surfaces, but he hesitated to conclude definitely that this organism was the cause of the disease, for he did not find it in all the cases of diphtheria examined, he was not able to produce paralytic phenomena in animals by its injection, and, further, he obtained the same organism from the throat of a healthy child. The organism became the subject of much inquiry, but its relationship to the disease may be said to have been definitely established by the brilliant researches of Roux and Yersin, which showed that the most important features of the disease could be produced by means of the separated toxin of the organism. Their experiments were published in 1888–90.

General Facts.—Without giving a description of the pathological changes in diphtheria, it will be well to mention the outstanding features which ought to be considered in connection

with its bacteriology. In addition to the formation of false membrane in the upper respiratory passages, which may prove fatal by mechanical effects, the chief clinical phenomena are the symptoms of general poisoning, great muscular weakness, tendency to syncope, and albuminuria; also the marked paralysis which occurs later in the disease, and which may affect the muscles of the pharynx, larynx, and eye, or less frequently the lower limbs (being sometimes of paraplegic type), all these being grouped together under the term "post-diphtheritic paralysis." It may be stated here that all these conditions have been experimentally reproduced by the diphtheria bacillus, or by its toxins. Other bacteria are, however, concerned in producing various secondary inflammatory complications in the region of the throat, such as ulceration, gangrenous change, and suppuration, which may be accompanied by symptoms of general septic poisoning. The detection of the bacillus in the false membrane or secretions of the throat is to be regarded as supplying the only certain means of diagnosis of diphtheritic infection, though it must be recognised that virulent or avirulent diphtheria bacilli may occur in the throat or nose unassociated with active or typical diphtheria, *e.g.* in carriers. On the other hand, fibrinous exudate may result from infection with other organisms, *e.g.* streptococci.

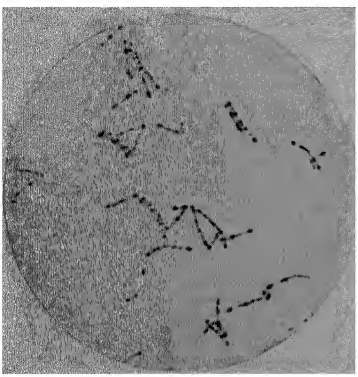


FIG. 59. — Diphtheria bacilli from a twenty-four hours' culture on agar. See also Plate III. Fig. 13. Stained with methylene-blue. ×1000.

Bacillus Diphtheriæ (*Corynebacterium diphtheriæ* (*Klebs-Löffler*)).—*Microscopical Characters.*—As seen in young cultures *B. diphtheriæ* occurs in the form of straight or, more frequently, slightly curved rods, which measure usually about 3 to 4 μ in length and 0·3 to 0·5 μ in thickness; both shorter and longer forms, however, are met with. The bacilli vary in configuration, being rounded or tapered at their ends, and they stain unequally, the staining frequently giving a sort of barred marking (Fig. 59). Mair has recently emphasised this "barred" staining as a feature

of certain strains of the organism. They contain granules which may produce a beaded appearance (Fig. 60) and which with certain dyes give a metachromatic reaction, for example, staining a purplish tint with polychrome methylene-blue. The granules are stained a deep, almost black, colour with Neisser's and other similar stains. They are usually situated at the poles of the bacilli—"polar granules"—but occur in other parts of the substance, and the longer bacilli may contain several. The ends of the bacilli are often expanded, especially in the longer forms; later these may form club-like structures which stain deeply, while the protoplasm becomes broken up into globules. Other bacilli may become thicker and segmented, and may even simulate a chain of cocci, and various stages of disintegration are seen (Fig. 61). These aberrant forms are usually regarded as the result of involution. Occasionally branched forms are met with. A characteristic feature in a film is the arrangement of the bacilli. They lie at various angles to one another, giving an appearance which has been compared to Chinese letters or cuneiform characters. This is apparently due to the snapping mode of division (p. 6); when a

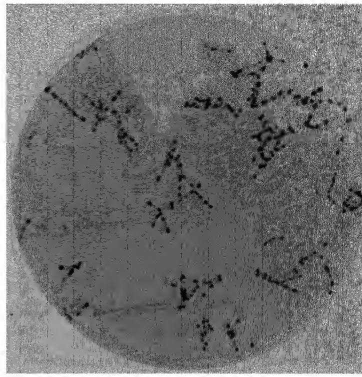


FIG. 60. — Diphtheria bacilli, showing beaded appearance due to deeply stained granules; from a twenty-four hours' culture on Löffler's serum. See also Plate III., Fig. 12.
Stained by Neisser's method. ×1000.

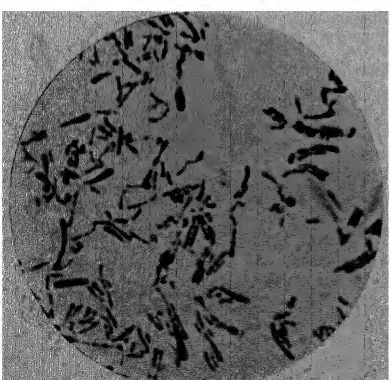


FIG. 61.—Involution forms of the diphtheria bacillus; from a forty-eight hours' culture on Löffler's serum. See also Plate III., Fig. 14.
Stained with methylene-blue. ×1000.

bacillus undergoes division the process at first occurs at one side and the two new individuals remain attached and set at an angle. It should be recognised that the size and general appearance vary with different strains of organisms and with different media, as well as with the duration of the growth. Sometimes quite short types are met with; rarely, in tryptic digest broth the culture may consist wholly of coccal forms arranged in clumps, diplococcal forms, and chains (Parish).

In films from the pharynx or from the membrane the bacilli have the same general characters, but they tend on the whole to stain more evenly and granules are not so pronounced (Fig. 64).

Staining.—They take up the basic aniline dyes, *e.g.* methylene-blue in watery solution, with great readiness, and stain deeply, the granules often giving the metachromatic reaction as described. They are Gram-positive, though they are rather more easily decolorised than the pyogenic cocci. When decolorised, however, the granules may still retain the Gram's stain. By Neisser's stain (p. 121) the bacilli are seen to contain granules stained almost black, the rest of the bacillary substance being coloured by the counter-stain (Plate III., Fig. 12). In applying the stain a Löffler's serum culture of eighteen to twenty-four hours' growth should be used. On other media metachromatic granules may not be demonstrable. The granules brought out by Neisser's method are often not visible in a methylene-blue preparation.

Neisser's stain is undoubtedly an important auxiliary in the recognition of the diphtheria bacillus, but the results of its use are to be interpreted with caution. Darkly staining granules are not peculiar to the diphtheria bacillus. Some cocci, often giving a metachromatic reaction with methylene-blue, may be stained deeply; and other bacilli may contain such granules. A not uncommon organism with such a character occurring in the throat is a strepto-bacillus with square ends; it has no resemblance to the diphtheria bacillus in a methylene-blue preparation, but when stained by Neisser's method may give an appearance very like that organism. On the other hand, a culture of Hofmann's pseudo-diphtheria bacillus (p. 401) reacts negatively with Neisser's stain: at most a few scattered granules may occur in the preparation, but the bacilli have not the beaded appearance. It will be found a good working plan to use the Neisser stain in parallel with methylene-blue. The stain is of special service in the case of the smaller forms of the diphtheria bacillus, the details of whose structure are imperfectly differentiated by methylene-blue. And again when the diphtheria bacilli are scanty they may be overlooked in a methylene-blue preparation, whereas they are more readily detected in a Neisser-stained preparation.

Most strains of the true diphtheria bacilli give the characteristic appearance with the Neisser stain when growing on such media as Löffler's serum, but it is of importance to observe that some hard waters interfere with the reaction. In such circumstances distilled water ought always to be used for washing the preparations.

Cultivation.—The diphtheria bacillus grows best in cultures at the temperature of the body; growth still takes place at 22° C., but practically ceases about 20° C. The best media are those which contain blood or serum, *e.g.* Löffler's original medium (p. 68), but growth occurs also on the ordinary agar media. If inoculations be made on the surface of coagulated serum with a piece of diphtheria membrane, colonies of the bacillus may appear in twelve hours, and are well formed within twenty-four hours, often before any other growths are visible. The colonies are small circular discs of opaque whitish colour, their centre being thicker and of darker greyish appearance than the periphery, when viewed by transmitted light. Their margins are at first regular, but later they become wavy or even crenated. On the second or third day they may reach 3 mm. in size, but when numerous they remain smaller. Their surface becomes distinctly granular, and when there is a mass of growth it comes to have a yellowish tint. On the agar media the colonies have much the same appearance, but grow less quickly, and sometimes they may be comparatively minute, so as rather to resemble those of the streptococcus group. In stroke cultures the growth forms a continuous layer of the same dull whitish colour, the margins of which often show single colonies partly or completely separated. Smooth and rough types of colonies have been distinguished (Cowan). In *gelatin* at 22° C. a puncture culture shows a line of dots along the needle track, while the growth at the surface is only slight. In none of the media does any liquefaction occur. Growth occurs under anaerobic as well as aerobic conditions. In *broth* the organism produces a turbidity which may settle to the bottom and form a powdery layer on the wall of the vessel. If the growth is started on the surface and the vessel is kept at rest, a distinct scum may form, and this is especially suitable for the development of toxin (*vide infra*).

Ordinary broth becomes acid during the first two or three days, and several days later again acquires an alkaline reaction which may then rise above the original. Changes in reaction are most pronounced when the medium contains sugars fermented by the organism, but even in sugar-free media reversal of the reaction occurs to some extent. This is due to formation of carbonates.

Peptone has a buffering action and diminishes the change to the acid side.

Further reference is made below to the cultivation of the diphtheria bacillus in relation to toxin production and to isolation of the organism from mixed cultures.

Two forms of the diphtheria bacillus, designated respectively *gravis* and *mitis*, have been distinguished by Anderson, Happold, McLeod and Thomson (Leeds), chiefly according to cultural characters; and they have associated these with different clinical types of the disease. *B. diphtheriæ gravis* occurs in severe toxic cases; it grows with granular deposit and pellicle in broth and has a flattened, lustreless colony of irregular

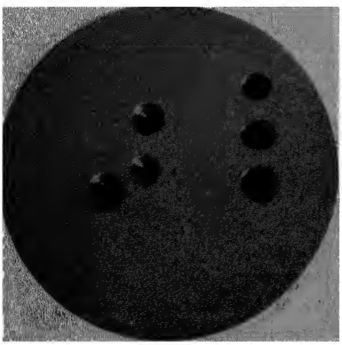


FIG. 62.—Colonies of *B. diphtheriæ mitis* on McLeod's medium (48 hours' growth). ×1½.

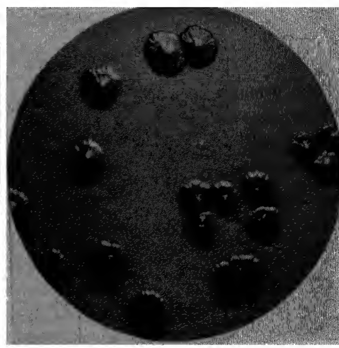


FIG. 63.—Colonies of *B. diphtheriæ gravis* on McLeod's medium (48 hours' growth. ×1½.

outline, which often exhibits a "daisy head" formation. *B. diphtheriæ mitis* is associated with cases in which general toxic symptoms tend to be slight, although there may be extensive false membrane; it grows with uniform turbidity in broth, and has a convex colony which is partly translucent. *Intermediate* forms also occur and may be regarded as forming a third biological type. The Park Williams No. 8 strain, which has been extensively used for toxin production, is of this nature. The cultural differences are best brought out on a special rabbit-blood tellurite medium (*vide* p. 75). These types differ also in their fermentative action on starch and glycogen (*vide infra*). The question of the significance of the biological types of the diphtheria bacillus will be discussed later.

Fermentative Properties.—The action of *B. diphtheriæ* in

fermentation has been extensively studied and is of considerable importance. It may be said that the organism produces acid *without gas formation* from glucose, maltose, and galactose, and no acid from lactose, saccharose, and mannitol. Glucose and saccharose are the sugars employed for practical purposes. In carrying out the tests it is important to ensure that there is abundant growth in the medium, and for this reason a serum medium such as Hiss's (p. 79), with 1 per cent. of a suitable peptone added is to be recommended, phenol red being used as indicator and the pH adjusted to 7·2. In Hiss's medium the development of acid is attended by the formation of clot. The fermentation of starch and glycogen has been regarded as a characteristic feature of the *gravis* form of the diphtheria bacillus, whereas the *mitis* and *intermediate* forms have no action on these polysaccharides. Reference to the fermentative properties of allied organisms is made below.

Hæmolysis.—The fact that certain strains of *B. diphtheriæ* may produce hæmolysis when grown in a blood medium has long been recognised. It has also been found that lysis may result when blood is added to fluid cultures. Whether this effect is due to a diffusible toxin similar to the hæmolysins of certain other bacteria, *e.g.* streptococci, remains doubtful. It may be noted that while the *mitis* type is usually hæmolytic, the *gravis* form usually lacks this property. The *intermediate* type is uniformly non-hæmolytic.

Powers of Resistance, etc.—In cultures the bacilli possess long duration of life; at room temperature they may survive for two months or longer. In the moist condition, whether in cultures or in membranes, they have a low power of resistance, being killed at 60° C. in a few minutes. On the other hand, in the dry condition they have great resisting powers. In membrane which is perfectly dry, for example, they can resist a temperature of 98° C. for an hour. Dried diphtheria membrane, kept in the absence of light and at the room temperature, has been proved to contain diphtheria bacilli still living and virulent at the end of several months. The presence of light, moisture, or a higher temperature causes them to die out more rapidly. Corresponding results have been obtained with bacilli taken from cultures and kept on dried threads.

Distribution of the Bacillus.—*B. diphtheriæ* may be found in the membrane wherever it is formed, and also occurs in the secretions of the pharynx and larynx in the disease. Virulent diphtheria bacilli have been found in a considerable proportion of cases of fibrinous rhinitis. In the case of any nasal

lesion, however, the test for virulence should always be made, as "diphtheria-like" bacilli without virulence are of comparatively common occurrence.

In diphtheria the membrane has a somewhat different structure, according as it is formed on a surface covered with stratified squamous epithelium, as in the pharynx, or on a surface covered by ciliated epithelium, as in the trachea. In the former situation necrosis of the epithelium occurs either uniformly or in patches, and along with this there is marked inflammatory reaction in the connective tissue beneath, attended by abundant fibrinous exuda-

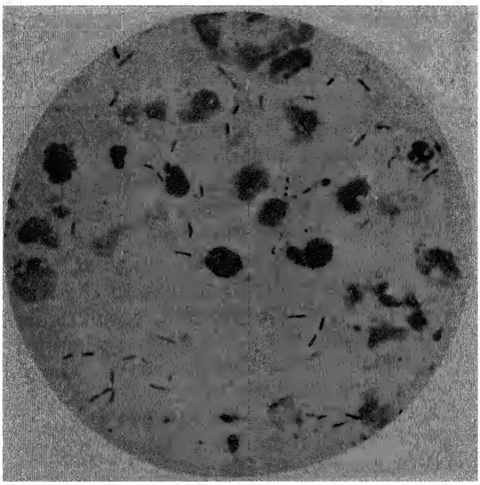


FIG. 64—Film preparation from diphtheria membrane, showing numerous diphtheria bacilli. One or two degenerated forms are seen near the centre of the field. (Cultures made from the same piece of membrane showed the organism to be present in practically pure condition.)
Stained with methylene-blue. ×1000.

tion. The necrosed epithelium becomes raised up by the fibrin, and its interstices are also filled by it; fibrinous exudation also occurs around the vessels in the tissue beneath. The membrane is thus firmly adherent, and when artificially detached it leaves a bleeding surface. In the trachea, on the other hand, the epithelial cells rapidly become shed, and the membrane is found to consist almost exclusively of fibrin with leucocytes, the former arranged in a reticulated or somewhat laminated manner, and varying in density in different parts The membrane lies upon the basement membrane, and is comparatively loosely attached.

The position of the diphtheria bacilli varies somewhat in different cases, but they are most frequently found lying in oval

or irregular clumps in the spaces between the fibrin, towards the superficial, that is, usually, the oldest part of the false membrane (Fig. 65). There they may be in a practically pure condition, though streptococci and occasionally some other organisms may be present along with them. They may occur also in deeper parts, but are rarely found in the fibrin around the blood vessels. On the surface of the membrane they may be also seen lying in large numbers, but are there accompanied by numerous other organisms. Occasionally a few bacilli have been detected in the

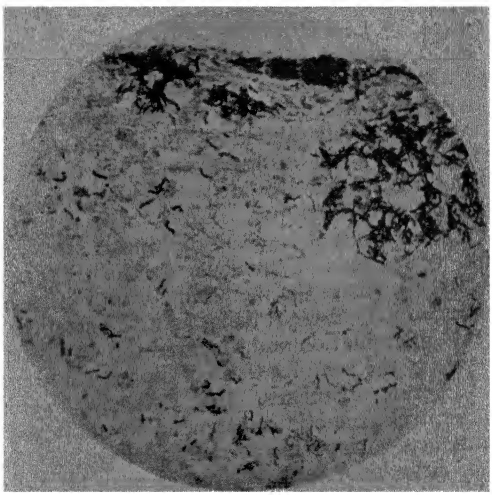


FIG. 65.—Section through a diphtheritic membrane in trachea, showing diphtheria bacilli (stained darkly) in clumps, and also scattered amongst the fibrin. Some streptococci are also shown towards the surface on the left side.
Stained by Gram's method. ×1000.

lymphatic glands. As Löffler first described, they may be found after death in pneumonic patches in the lung, these being due to a secondary extension by the air passages. They have also been occasionally found in the spleen, liver, and other organs after death; in a few instances they have been cultivated from the blood during life. Such occurrences are probably to be explained by an entrance into the blood stream shortly before death. It may be accepted, however, that the growth of the organism is essentially local, that it does not invade the

tissues to any extent, and that its important effects are produced by toxins absorbed from the site of growth. *B. diphtheriæ* may also infect other mucous membranes; thus diphtheria of the conjunctiva has been found occasionally, and similar affections of the vulva and vagina may also occur. In most of the recorded cases of such lesions, however, the identity of the bacilli has not been established; some of them are described as responding to treatment with antitoxin. During the war virulent diphtheria bacilli were cultivated from wounds in a small proportion of cases; their presence was not attended by any special effects.

Association with other Organisms.—In diphtheria the pyogenic organisms—streptococci, staphylococci, pneumococcus, etc.—are practically always present in the pharynx, occurring there in varying numbers and combinations. Hæmolytic streptococci are, however, relatively abundant in certain cases and can hardly be without effect in aggravating the condition. They are often found lying side by side with the diphtheria bacilli in the membrane, and also penetrating more deeply into the tissues. In some cases of tracheal diphtheria streptococci have been found alone at a lower level in the trachea than the diphtheria bacilli, where the membrane was thinner and softer, the appearance in these cases being as if the streptococci acted as exciters of inflammation and prepared the way for the bacilli. Further, some of the complications of diphtheria are due to the action of pyogenic organisms. The extensive swelling of the tissues of the neck, sometimes attended by suppuration in the glands, and also various hæmorrhagic conditions, have been found to be associated with their presence; the diphtheritic lesion enables them to get a foothold in the tissues, where they exert their usual action and may lead to extensive suppurative change, or even to septicæmia. In cases where a gangrenous process is superadded, a great variety of organisms may be present, some of them being anaerobic. Against such complications produced by other organisms anti-diphtheria serum has no effect.

Effects of Inoculation.—In considering the effects produced in animals by experimental inoculations of pure cultures, we have to keep in view the local changes which occur in diphtheria, and also the symptoms of general poisoning.

As Löffler stated in his original paper, inoculation of the healthy mucous membranes of various animals with pure cultures causes no lesion, but the formation of false membrane may result when the surface is injured by scarification or otherwise.

A similar result may be obtained when the trachea is inoculated after tracheotomy has been performed. The membrane produced by such experiments is usually less firm than in human diphtheria, and the bacilli in the membrane are less numerous. Rabbits inoculated after tracheotomy often die, and Roux and Yersin were the first to observe that in some cases paralysis might appear before death.

Subcutaneous injection in guinea-pigs of diphtheria bacilli in a suitable dose produces death within thirty-six hours. At the site of inoculation there is usually a small patch of greyish membrane with some necrosis, whilst in the tissues around there is extensive inflammatory œdema, often associated with hæmorrhages, and there is also swelling of the corresponding lymphatic glands. The internal organs show general congestion, the suprarenal capsules being especially reddened and often hæmorrhagic. The renal epithelium may show cloudy swelling, and there is often effusion into the pleural cavities. After injection the bacilli increase in number for a few hours, but multiplication soon ceases, and at the time of death they may even be less numerous than when injected. The bacilli remain practically local, cultures made from the blood and internal organs usually giving negative results. If a non-fatal dose of a culture be injected, a local necrosis of the skin and subcutaneous tissue may follow at the site of inoculation.

In rabbits, after subcutaneous inoculation, results of the same nature follow, but these animals are less susceptible than guinea-pigs, and the dose requires to be proportionately larger. Roux and Yersin found that after intravenous injection the bacilli rapidly disappeared from the blood, and when 1 c.c. of a broth culture had been injected no trace of the organisms could be detected by culture after twenty-four hours; nevertheless the animals died with symptoms of general toxæmia, nephritis also being often present. The dog and sheep are also susceptible to inoculation with virulent bacilli, but the mouse and rat enjoy a high degree of immunity.

An *intracutaneous method* of injection has been found to be of service in testing the virulence, and thus in the identification, of the diphtheria bacillus, especially when used in conjunction with the injection of antitoxin. Injection with a fine syringe of a small amount of diphtheria bacilli into the superficial part of the skin in a guinea-pig produces a circumscribed swelling which is followed by superficial necrosis in from one to two days; whereas, if the animal has received previously an injection of, say, 250 units of antitoxin, the result is negative. The result

is also negative (without antitoxin) in the case of an avirulent diphtheroid (p. 396). This test can also be carried out on the rabbit.

Toxins of Bacillus Diphtheriæ.—As in the above experiments the symptoms of poisoning, and ultimately a fatal result, occur when the bacilli are not increasing in number, or even after they have practically disappeared, Roux and Yersin inferred that the chief effects were produced by toxins, and this supposition they proved to be correct. They showed that broth cultures of three or four weeks' growth freed from bacilli by filtration were highly toxic. The filtrate when injected into guinea-pigs and other animals produces practically the same effects as the living bacilli; locally there is little fibrinous exudation but a considerable amount of inflammatory œdema, and, if the animal survives long enough, necrosis of the superficial tissues in varying degree may follow. The toxicity may be so great that 0·002 c.c. or less may be fatal to a guinea-pig in five days. One point of much interest is the high degree of resistance to the toxin possessed by mice and rats. Roux and Yersin, for example, found that 2 c.c. of toxin, which was sufficient to kill a rabbit in sixty hours, had no effect on a mouse, whilst of this toxin even $\frac{1}{15}$ c.c. produced extensive necrosis of the skin of the guinea-pig.

After injection either of the toxin or of the living bacilli, when the animals survive long enough, a state of marasmus sets in or paralytic phenomena occasionally occur. In the latter condition the hind-limbs are usually affected first, the paralysis afterwards extending to other parts, though sometimes the fore-limbs and neck first show the condition. Sometimes symptoms of paralysis do not appear till two or three weeks after inoculation. After paralysis has appeared, a fatal result usually follows in the smaller animals, but in dogs recovery may take place. It is to be noted in this connection that paralytic symptoms are of not uncommon occurrence in the human subject after treatment with antitoxin. The production of these paralytic phenomena was ascribed by Ehrlich to *toxone*, as they specially occur when there is injected along with the toxin sufficient antitoxin to neutralise the more rapidly acting toxin proper. This toxone was supposed to have a different toxophore group (p. 198) from that of the ordinary toxin, so that it produced the late nervous phenomena, while its local action on the tissues was very slight. Owing to its weaker affinity for antitoxin, much of it might be left unneutralised. Recent work of Prigge, however, indicates that toxone has

no separate existence as a constituent of diphtheria toxin, since he was able to reproduce the paralytic effects regularly in guinea-pigs by repeated injections of minute doses of diphtheria toxin—without any admixture with antitoxin—over a long period. Thus paralysis is the result of a *chronic* intoxication with the products of the diphtheria bacillus.

Preparation of the Toxin.—The obtaining of a very active toxin in large quantities is an essential in the preparation of anti-diphtheritic serum. Certain conditions favour the development of a high degree of toxicity, namely, ***a free supply of oxygen, the presence of a large proportion of peptone or products of tryptic digestion in the medium, the absence of substances which produce an acid reaction, and an initial reaction of pH*** 7·5–8. In the earlier work a current of sterile air was made to pass over the surface of the medium, as it was found that by this means the period of acid reaction was shortened and the toxin formation favoured. This expedient is now considered unnecessary if an alkaline medium free from glucose is used, and the cultures are made in shallow layers of medium. It was recognised by the early workers that the occurrence of a surface pellicle of growth favoured toxin production and this has recently been emphasised by Pope and Healey. The absence of glucose may be attained by fermenting the meat infusion with yeast. L. Martin introduced a medium composed of equal parts of freshly prepared peptone (by digesting pigs' stomachs with HCl at 35° C.) and glucose-free veal broth. With this medium he obtained a toxin of which 0·002 c.c. was the fatal dose to a guinea-pig of 500 grams. A medium prepared from a tryptic digest of horse flesh, which possesses advantages and has been much used in recent years is Hartley's broth (p. 58). As a constituent of medium for toxin production commercial peptones vary considerably and a specially prepared tryptic digest provides the most satisfactory basis. Certain organic substances have been found to be valuable in augmenting the formation of toxin, *e.g.* sodium acetate, sodium lactate and maltose (Pope and Healey). It may be noted that maltose is only slowly converted to glucose by the growth of the organism and its presence does not lead to excessive acid production. A medium fulfilling these various requirements is that of Pope and Smith. It incorporates a glucose-free meat infusion, a tryptic digest of meat, 0·3 per cent. maltose, and 0·1 per cent. sodium lactate. Such culture media apparently contain valuable factors for toxin production which are heat-labile, *e.g.* at the temperature of the autoclave, and therefore it is advisable to sterilise by filtration through a Seitz filter followed by ten minutes steaming at 100° C. As emphasised above, the reaction of the media should be accurately adjusted within the limits mentioned (pH 7·5–8). There is in all cases a period at which the toxicity reaches a maximum; Roux and Yersin found this period to be two to three weeks, but by later methods the greatest toxicity is reached about the tenth day or even earlier. An important factor also is the oxygen tension which depends on the relative area of surface of the medium exposed to the air; thus toxin formation occurs most rapidly in shallow layers of medium,

e.g. in large flat bottles laid on their sides. When maximum toxicity has been reached the clear fluid is decanted from the sediment and covered with a layer of toluol; it is then shaken on several occasions for two or three days. When toxin is to be used for testing purposes it is kept in an ice-chest, for about twelve months, till it has been "ripened" or stabilised. (Some workers filter the decanted fluid.) It may be added that the power of toxin formation varies much in different strains of the diphtheria bacillus.

Properties and Nature of the Toxin.—The toxic substance in filtered cultures is a relatively unstable body. When kept in sealed tubes in the absence of light, it may preserve its powers little altered for several months, but, on the other hand, it gradually loses them when exposed to the action of light and air. As has been shown, the toxin probably does not become destroyed, but its toxophore group suffers a sort of deterioration, so that a toxoid is formed which has still the power of combining with antitoxin (p. 198). Heating at 58° C. for two hours destroys the toxic properties in great part, but not altogether. When, however, the preparation is evaporated to dryness, the toxin has much greater resistance to heat. One striking fact, discovered by Roux and Yersin, is that after an organic acid, such as tartaric acid, is added to the toxin the toxic property disappears, but it can be in great part restored by again making the fluid alkaline.

Toxic bodies have also been obtained from the tissues of those who have died from diphtheria. Roux and Yersin, by using a filtered watery extract from the spleen in very virulent cases of diphtheria, produced in animals death after wasting and paralysis, and also obtained similar results by employing the urine.

Variations in Virulence and in Toxin Production.—The distinction between virulence—the power to invade the tissues and produce disease—and the faculty of toxin production has already been emphasised (p. 180); it is well illustrated in the case of *B. diphtheriæ*. For comparative tests of virulence known quantities of bacilli from a young culture on solid medium should be used for injecting a guinea-pig, as in fluid cultures the presence of variable amounts of toxin modifies the result. As shown by the amount of culture necessary to produce death of a guinea-pig, it may be said that all degrees of virulence are met with among freshly cultivated strains. The virulence of a given strain, moreover, is usually well maintained in culture. A certain falling off has sometimes been observed after a time. Further, unlike what obtains with other organisms, little or no increase of virulence can be ob-

tained by means of *passage*. The earlier results of Roux and Yersin, according to which after an attack of diphtheria the bacilli in the throat gradually become attenuated and ultimately non-virulent, have not been substantiated; and conversely, no one has succeeded in transforming a non-virulent diphtheria bacillus into a virulent one. It is now generally accepted that virulence is a relatively stable property. In the usual subcutaneous test for virulence (p. 161) a whole growth on a serum slope culture is used, and either death of the animal or no pathogenic effect is the common result. Some non-virulent strains have been found, however, to produce a small amount of toxin in media or to give rise to a small amount of antitoxin on injection. These facts indicate that all gradations are found, and rather suggest that the non-virulent organisms belong to the same species as the virulent.

A certain correspondence, however, exists between virulence and toxin production, inasmuch as every virulent strain produces toxin, but the two properties do not run in a parallel manner, as some strains of moderate virulence are very active in toxin production. In the preparation of antitoxins the obtaining of a very powerful toxin is necessary, and for this purpose many strains may have to be tested before a satisfactory toxin producer is obtained. A strain obtained by Park and Williams many years ago, known as No. 8, has been used very extensively all over the world and is noted for its properties in this respect. This is an illustration of the well-recognised fact that the power of toxin production usually remains unchanged for a long period in conditions of artificial cultivation; the strain, however, is not highly virulent. Cultures derived originally from a single diphtheria bacillus have yielded both virulent and non-virulent strains; and Cowan has isolated from a virulent culture, by repeated plating, colonies of "rough" type consisting of organisms which were non-virulent and did not produce toxin. Also the observations made by Okell on virulent and avirulent strains isolated from the same patient and from different patients in the same epidemic suggest that *in vivo*, virulent bacilli may occasionally yield avirulent variants. It has been stated that the diphtheria bacilli recovered from cases in which antitoxin treatment has failed, are characterised by their high virulence.

The significance of the biological types of the diphtheria bacillus.—When the *gravis* and *mitis* types were first distinguished it was pointed out that a high correlation existed between the clinical severity of the disease and the cultural type, the *gravis* form being

associated with cases of diphtheria of unusual severity which exhibited a relative refractoriness to antitoxin treatment. The Leeds workers found that 95 per cent. of strains belonged to these types, though a small percentage were classified as " intermediate." Later studies have all shown that this *intermediate* form occupies a very prominent position among the three types (Robinson and Marshall; Wright and Christison; Carter; Murray; Cooper *et al.*). The main characters on which differentiation has been based are the colony features (*vide* p. 378) and the fermentation of polysaccharides, *e.g.* starch, the *gravis* type fermenting this substance, while the *mitis* and *intermediate* are inactive. It is unnecessary here to refer to the various other differential features, but it has been emphasised that virulence for animals is a constant character of the *gravis* strains; the *intermediate* are virulent with few exceptions; the *mitis* strains are usually virulent but vary in this respect. In addition to the original types, Wright and Christison have described three others all exhibiting a *gravis* form of colony but differentiated according to starch fermentation and virulence and urge a numerical denomination of types. J. Wright has added a seventh type. These are shown in the following table:

	Colony Form.	Starch Fermentation.	Virulence.
Type I.*	*Mitis* . .	−	+
Type II.*	*Intermediate* .	−	+
Type III.*	*Gravis* . .	+	+
Type IV.	,, . .	−	+
Type V.	,, . .	+	−
Type VI.	,, . .	−	−
Type VII.	*Mitis* . .	+	+

* Types originally described by Anderson *et al.*

Wright and Christison found, moreover, a small residuum of unclassified strains. They point out that in Edinburgh a considerable proportion of the strains do not conform to the characters of *gravis*, *mitis*, or *intermediate*. In Edinburgh (1932–1937) the majority of strains (though few in number) which are identical with the *gravis* type, have been isolated from mild and carrier cases.

It has emerged from work in various parts of the country that the prevalence of types, while in some areas corresponding to that in Leeds, has differed in others. Thus in some places the " intermediate " type has predominated and the *gravis* type has been comparatively rare. Continuous study over several years has also shown that the relative prevalence has altered in the course of time. From an extensive survey, Cooper, Happold, McLeod and Woodcock have concluded that *gravis* infection is more formidable than *mitis* infection, but that the *intermediate* strains approximate in pathogenicity for the human subject more nearly to *gravis* than *mitis*. It is admitted, however, that all three types may be met with in phases of exalted or diminished virulence. While there is evidence that the *gravis* type at present is associated with

a more severe form of the disease than the *mitis*, it is difficult to draw any clear distinction between *gravis* and *intermediate* type infections and it remains undecided how much clinical and epidemiological significance should be attached to the occurrence of these types. The question has also arisen as to their stability. It is now generally agreed that colony-form is an unstable character and that strains change in this respect. Though fermentation of starch has appeared to be more stable, variation in this property has also been observed (see Robinson; Mair). Hammerschmidt has stated that there may be a complete change from the *intermediate* to the *mitis* and *gravis* types. However, it has been claimed that there is no antigenic relationship between the types (Ewing, Murray). In view of the refractoriness to antitoxin treatment of the cases infected by the *gravis* type, the question has arisen whether this form might produce a different toxin from the other types. No such immunological difference has been detected, however.

It is manifest from all this work that, as might be expected, strains of the diphtheria bacillus may differ quantitatively in their virulence for the human subject and that such differences in virulence may be associated with differences in biological characters. On the other hand, further continuous observations over a number of years will be required to determine whether different grades of pathogenicity are associated with well defined and fixed biological types of the organism.

Diphtheria Carriers.—It has been known for some time that *B. diphtheriæ* may persist for considerable periods in the throats of those who have suffered from the disease. As a rule, it disappears within a few days after recovery, but in a small proportion of cases it may be found for several weeks or even months afterwards. Such individuals harbouring the bacilli are known as "convalescent carriers." The bacillus may also be found in the throats of individuals who have been in contact with patients or other carriers—"contact carriers," and accordingly these also may spread infection. Some of the carriers suffer from slight indisposition, sore throat, etc., but others have no clinical symptoms at all. It is generally admitted that the persistence of the bacilli is aided by abnormal conditions of the fauces, and the treatment of these is thus of importance. The bacilli have been found, for instance, in the crypts of enlarged tonsils, and the removal of the latter may be attended by favourable results. The carrier condition may be also due to the presence of bacilli in the nasal cavity, and here it may or may not be accompanied by morbid states. In all cases, apart from the presence of clinical diphtheria, when an organism possessing the morphological and cultural characters of the *B. diphtheriæ* has been isolated the test for virulence must be made. It is now generally recognised that the avirulent types play no part

in the spread of the disease, and that individuals harbouring them are not a danger to the community. Moss, Guthrie, and Marshall have found that the carrier state (without pathogenic effects) is easily produced by inoculating the throat of healthy persons with non-virulent diphtheria bacilli. Prior administration of antitoxin did not prevent the development of the bacilli in the throat. The carrier state so produced tended to persist and there was no evidence of any change in the characters of the organisms, which remained non-virulent. Further, no cases of clinical diphtheria occurred among the associates of such carriers. These workers have also recorded experiments in which Schick-positive and -negative persons were inoculated with a virulent culture of the diphtheria bacillus; typical clinical diphtheria resulted in the positive reactors, whereas the negative reactors became carriers without showing clinical effects. As a rule, chronic carriers give a negative Schick reaction, that is, are immune (*vide infra*). In the investigation of healthy contact carriers the Schick test accordingly adds valuable information when a suspicious organism has been cultivated from the fauces. Thus, if the suspected carrier is Schick-positive, *i.e.* non-immune, the organism is likely to be non-virulent.

In one or two exceptional instances, diphtheria bacilli have been obtained from the lower animals, *e.g.* cow and horse, but there is no evidence that animal infection plays any part in the spread of the human disease. It may also be added that the so-called "diphtheritic" diseases of animals are due to quite different organisms.

Immunity.—This is described in the general chapter on Immunity. It is sufficient to state here that a high degree of immunity, against both the bacilli and their toxins, can be produced in various animals by gradually increasing doses either of the bacilli or of their separated toxins. As a result of the immunisation, antitoxins appear in the serum, and these are capable of protecting animals either against infection with diphtheria bacilli or against their toxins. They also have curative effects in animals which are already the subjects either of infection or intoxication. The production and standardisation of diphtheria antitoxin for medical use are dealt with on p. 214 *et. seq.*

Reference has been made (p. 215) to the fact that in a certain proportion of horses in the natural state antitoxin is present in the blood, and a similar statement also holds with regard to the human subject. The presence of the antitoxin is shown by mixing a certain amount of the serum with a small quantity of

toxin and injecting some of the mixture intracutaneously in the guinea-pig (Römer's test); an injection of the same amount of toxin alone is made at another spot as a control. The application of this test gave important knowledge with regard to the presence of antitoxin at different ages, but for practical purposes it has been superseded by the Schick test, which may now be described. The presence of $\frac{1}{30}$ to $\frac{1}{100}$ unit of antitoxin per cubic centimetre of serum is probably sufficient to afford the individual protection against infection.

Schick Test.—This test depends upon the fact that when a minute amount of diphtheria toxin is introduced into the skin an inflammatory reaction results, provided that insufficient antitoxin is present in the blood to neutralise the effects. As has just been stated, however, the blood of a certain number of individuals contains a small though sufficient amount of antitoxin, and in these the reaction does not occur—the result is negative. The presence of antitoxin in the blood is attended by a certain amount of immunity, and thus the test gives an indication of the presence or absence of immunity, according as the result is negative or positive respectively. The amount of toxin injected is of importance; it should be sufficient to ensure a positive reaction in an individual without antitoxin, and it should not be so great as to be in excess of the amount of antitoxin supposed to be associated with immunity. The dose of toxin used is essentially that amount which is just neutralised by $\frac{1}{1000}$ unit of antitoxin. For the purpose an old toxin preparation is usually selected in which some of the toxin has undergone alteration to toxoid, and this is diluted with a buffer solution [1] so that 0·2 c.c. of the dilution contains the test-dose. This buffered preparation can be kept for some time without deterioration. Occasionally there occur pseudo-reactions of the nature of a supersensitiveness or allergy, which are due to certain substances other than the toxin in the filtrate, this allergy being met with alike in immune and non-immune subjects. Diphtheria toxin is readily destroyed by heating, whereas the substances leading to pseudo-reactions are not. A similar preparation containing the same concentration of toxin heated to 75° C. for five minutes can thus be used as a control, and of course any effect produced by it will be of the nature of a pseudo-reaction, that is, will not be due to the true toxin. Test and control preparations for carrying out this test are now supplied by various firms.

[1] The solution contains borax, boric acid, and sodium chloride, with Witte's peptone or human serum as a stabiliser.

The test is made by intracutaneous injection on the flexor aspect of the forearm, one arm being used for the toxin, the other for the heated toxin as a control (p. 162). In the case of a positive reaction (in a non-immune individual without pseudo-reaction) an area of redness and slight swelling appears after twenty-four to forty-eight hours, and reaches its maximum about the fourth day, when it may measure up to 2 cm. or more in diameter. It persists for seven days and then gradually fades, becoming brownish in tint, and there is usually some desquamation of the surface epithelium. The control injection on the other arm is, of course, attended by no result. If the reaction is negative, no change follows at the site of injection of the diluted toxin, the condition of the two arms being similar.

A pseudo-reaction is indicated also by an area of redness, but it appears earlier, often after six hours, is less intense and less definite at its margins than the positive reaction, and usually disappears in from one to three days. If it is associated with a negative Schick reaction, the result will be the same on the two arms. If a positive Schick reaction is present, the effect due to the unheated toxin will be more marked and persist longer than the reaction on the control arm. The difference is, as a rule, specially marked on the fourth to seventh day, when the reading should be taken; a positive reaction is then still present, while a pseudo-reaction is quite gone. Pseudo-reactions are most frequently met with in persons over six years of age, and in them a control is absolutely necessary.

Extensive series of observations at different periods of life have been carried out by the Römer and by the Schick method, and the results correspond on the whole fairly closely. It may be said that, at the time of birth, the serum of 80 per cent. of children contains antitoxin, and thus they give a negative Schick reaction, that this number falls for a year or two, at first rapidly, and that then there is a gradual rise until in adult life the original level is reached. The age of maximum susceptibility may be said to be from one to four years. It is generally agreed that the antitoxin of the infant represents a passive immunity transferred from the mother, hence the percentages of mothers and of infants whose serum contains antitoxin, correspond. This passive immunity falls off somewhat rapidly, and thus a greater proportion of Schick-positive reactions occur. The gradual diminution of the number of the latter which then follows is supposed to be due to a process of active immunisation occurring as the result of the individuals harbouring diphtheria bacilli and of slight infections by the organism. This, however,

may not be the complete explanation, since observations on isolated communities have indicated that although diphtheria infection is absent, the proportion of Schick-negative reactions increases with age much as in Europe. Accordingly the possibility must be considered that there is a natural development of diphtheria antitoxin.

Prophylactic Immunisation.—This was originally carried out by active immunisation with a small amount of toxin partly neutralised by antitoxin, the amount of the mixture used for injection (*e.g.* 1 c.c.) being only slightly toxic to a guinea-pig. The method was applied on a large scale by Park, Zingher, and others, and the results proved distinctly favourable. Three consecutive injections were given with an interval of a week between, and the immunisation was recommended to be carried out in young children from about a year old, so as to give protection during the highly susceptible period of life. Immunity, as shown by a previously positive Schick reaction becoming negative, developed somewhat slowly, but in 90 per cent. of the cases the reaction became negative after three months. Observations were carried out on 200,000 school children in America; half of these were tested for the Schick reaction, and those giving a positive reaction were immunised, the other half were used as controls. The result was that amongst the controls the number of cases of diphtheria which occurred was five times as great as amongst those tested and treated. Further, all the severe cases occurred amongst the controls (Park and Williams).

Immunisation with Toxoid.—More recently for purposes of active immunisation use has been made of the toxoid introduced by Ramon, also called anatoxin, which results from the action of formalin on diphtheria toxin. Glenny and his co-workers have found, however, that on account of its rapid elimination, toxoid does not provide a continuous stimulus, but when it is converted into a relatively insoluble precipitate even more effective antigenic action is secured. This may be done by precipitation of the toxoid with potash alum; a similar effect is obtained by using the floccules which result when toxoid is mixed with antitoxin. Toxin is converted to toxoid by the addition of 0·3 per cent. formalin and keeping the preparation for three weeks at 37° C. The final product is controlled by injecting 5 c.c. subcutaneously into a guinea-pig and ascertaining in this way that it is non-toxic.

Various preparations of toxoid have now been applied practically for prophylaxis: (1) formol-toxoid (F.T.); (2) alum-treated toxoid (Al.F.T.); (3) toxoid-antitoxin mixture (T.A.M.)

containing toxoid partially neutralised by antitoxin, and (4) toxoid-antitoxin floccules (T.A.F.) which is a suspension of the flocculent precipitate produced when toxoid and antitoxin are mixed in neutralising proportions.[1] Formol-toxoid and alum-toxoid are highly effective antigens, but have a tendency to cause troublesome local and general reactions when administered to older children and adults. This can be predicted in the individual case by a preliminary test originally advocated by Moloney and Fraser—the "Moloney test"—in which a minute amount of the toxoid is injected intradermally at the same time as the preliminary Schick test is carried out. The occurrence of a local skin reaction indicates that the individual is sensitive and if so the dosage of the prophylactic should be reduced or an alternative preparation employed. Both the toxoid-antitoxin mixture and the toxoid-antitoxin floccules are much less likely to produce these reactions. Of these the latter (T.A.F.) is the more effective antigen and should in fact be used in preference to ordinary toxoid in older children and adults, especially if a preliminary test with toxoid reveals sensitiveness. This prophylactic is, however, more difficult to prepare and is somewhat more costly. In general, the dose of these preparations is 1 c.c. administered on three occasions at intervals of two to four weeks, the injection being made intramuscularly in the upper arm. In all cases the resulting immunity should be controlled by a subsequent Schick test which should be negative if the prophylaxis has been effective. Generally the reaction is found to be negative four to twelve weeks after immunisation

Active immunisation with toxoid has been found highly effective in controlling the incidence of diphtheria among the nursing and domestic staffs of fever hospitals.

Therapeutic Effects of Diphtheria Antitoxin.—The use of this antitoxin for the prevention and treatment of diphtheria constituted the first great contribution of bacteriology to practical therapeutics. The practice as to method of administration and dosage still varies, but larger doses are now given than formerly, and it is generally recognised that the best routes of injection are the intramuscular and intravenous. The former is the more convenient, but the latter gives the more rapid distribution of antitoxin throughout the body and is thus more effective. Further, the longer treatment has been delayed or the severer

[1] *The Therapeutic Substances Regulations*, prescribe tests which ensure that commercial supplies of "Diphtheria Prophylactic" (toxin-antitoxin. toxoid, etc.) shall not be too toxic, and at the same time shall possess efficient immunising properties.

the case, the more is the intravenous method to be preferred and the larger should be the dose. Subcutaneous injection is much less efficient, as the absorption of antitoxin into the circulation takes place relatively slowly. Some authorities give the antitoxin in a single dose, others in two or more doses. Theoretically, there appears to be no advantage in the latter method, as a single dose will keep the antitoxin content of the blood during the disease at a level more than sufficient to neutralise the toxin; nevertheless, some maintain that administration in more than one dose has an advantage. The dose, of course, varies also according to the age of the patient, but for a child is proportionately larger than for an adult. We may say that the average dosage recommended is, for an infant, 2000–10,000 units, varying according to the principles mentioned above, and for an adult, 5000–50,000 units. In some places, however, the practice is to give even larger amounts. Thus in cases with extensive false membrane Bie injects the serum both intravenously and intramuscularly; for a child under ten years of age an initial dose of 80,000 units is given (a quarter to a third of which is injected intravenously), followed at intervals of twelve to twenty-four hours by two further intramuscular doses, the total amounting to 160,000 units or more. It is claimed that in very severe cases the results of treatment are much improved by such dosage. Without stating the details required for an adequate discussion of the results of antitoxin treatment, it may be said that statistics obtained from all parts of the world afford convincing proof of its advantage. And this is reflected in the practically universal belief of the medical profession in its efficiency. In recent years there has occurred with increased frequency a severe form of diphtheria which responds poorly to antitoxin treatment. According to the observations of Anderson, Happold, McLeod, and Thomson in Leeds, the intractable cases are due to infections with the *gravis* form of the bacillus (p. 389).

Other Antibodies.—As in the case of other organisms, the injection into animals of *B. diphtheriæ* itself in the living or dead condition, gives rise to antibacterial substances—agglutinins, complement-fixing bodies, etc. Some of these may be present in antitoxic sera, due to the presence of disintegration products of the bacilli in the culture fluid used for preparing the antitoxin; but there is no evidence that such substances play any part in the therapeutic effects.

Serological types.—It is now clear that diphtheria bacilli present considerable heterogeneity as regards their serological reactions

and antigenic constitution. On the other hand, all strains yield the same toxin. The serology of the organisms, as apart from their toxins, has been studied mainly by agglutination tests with antisera and recently particular attention has been paid to the reactions of strains classified in the first place as *gravis*, *mitis*, and *intermediate*. Agglutination tests are complicated by the difficulty of obtaining uniform suspensions of the organisms and special methods have been adopted to overcome this difficulty (see Orr-Ewing; Murray; Robinson and Peeney). The results of recent workers show that even within the types *gravis*, *mitis*, and *intermediate* there is serological heterogeneity. Robinson and Peeney have recognised by agglutinin-absorption tests five subtypes of the *gravis* form. They point out that a strain may vary in colony form and starch-fermenting capacity without alteration in serological reactions; further, that virulent and avirulent strains cannot be distinguished on a serological basis. It is of special interest that no serological relationship has been elicited between the three cultural types, *gravis*, *mitis*, and *intermediate* (Murray). While a particular serological variety may be associated with a particular epidemic, serological classification so far has not proved of any practical significance.

Identification of the Diphtheria Bacillus—Allied Organisms

It is now recognised that *B. diphtheriæ* is a member of a group of organisms with closely allied characters, which are of common occurrence and have a wide distribution as commensals of the skin and mucous membranes of man and the lower animals. This groups corresponds with the genus *Corynebacterium* of the Association of American Bacteriologists, and its characterisation as modified by a Committee of the Medical Research Council may be stated as follows:

"Gram-positive rod-like forms, arranged usually in a palisade, not acid-fast, often with club-shaped swellings at the poles, generally with irregularly stained segments or granules, non-motile, without endospore formation, growing aerobically or under micro-aerophilic conditions, but often capable of anaerobic cultivation, never forming gas in carbohydrate media, in which they may or may not produce acidity; they may or may not liquefy gelatin or serum."

Members of the genus *Corynebacterium* other than the diphtheria bacillus are usually known in this country as "diphtheroids." These have been obtained from the mouth, ear, nose, skin, genital organs, and even from the blood in certain diseases. They are to be met with in conditions of health, and they have been obtained from many diverse morbid conditions—from skin diseases, from coryza, from leprosy, from

gun-shot wounds, and even from general paralysis of the insane; the acne bacillus also belongs to this group. As has been found with other groups, the differentiation is a matter of considerable difficulty. Some are practically identical with the diphtheria bacillus both morphologically and culturally, and give the characteristic reaction with Neisser's stain; others, again, differ in essential particulars. The fermentative action on sugars (p. 378) has also been called into requisition as a means of distinguishing them, but the results obtained cannot be said to be of a decisive character. The absence of the power of fermenting glucose or the presence of capacity to ferment saccharose may, however, be accepted in any particular case as sufficient to exclude the organism from being the diphtheria bacillus. Certain species of diphtheroids produce pseudo-tuberculosis in sheep and mice, ulcerative lymphangitis of horses, and pyogenic lesions in cattle and pigs; but in general the diphtheroids obtained from the human body are not responsible for any specific pathogenic effects. Further, cultures of these organisms are as a rule quite non-virulent when tested by inoculation of guinea-pigs.

Cases have been recorded, however, in which diphtheroid bacilli have been specifically associated with disease in the human subject. Gilbert and Stewart have described three outbreaks of throat infection, apparently milk-borne, traced to carriers of a diphtheroid bacillus designated *Corynebacterium ulcerans*.

Schultz *et al.* have reported a case of meningitis due to a diphtheroid bacillus (*Cornyebacterium parvulum*). Gibson has also isolated a similar organism from a fatal case of meningitis, which appeared to be the specific etiological agent. The cellular exudate in the cerebro-spinal fluid was mainly mononuclear. The bacillus did not exhibit metachromatic granules by Neisser's staining method, and in cultural characters it also differed from the diphtheria bacillus. Exotoxin production could not be demonstrated, but the organism was virulent for various laboratory animals. Gram-positive bacilli classified with *Bacterium monocytogenes* (*vide* p. 403) have also been reported in meningeal infections and the question of the relationship of these diphtheroid bacilli to *B. monocytogenes* requires consideration.

Barratt has described a group of "diphtheria-like" organisms isolated from the human naso-pharynx which, while resembling the diphtheria bacillus, differ from it in certain features and particularly their capacity to liquefy gelatin. They would

appear to have some biological relationship to the Preisz-Nocard bacillus (*vide infra*). These organisms are virulent to guinea-pigs but their effects are not neutralised, or only partially so, by diphtheria antitoxin. They differ also from the diphtheria bacillus in being pathogenic to rats.

We give below an account of the characters of certain well-defined types of diphtheroid bacteria.

From what has been said it will be clear that the scientific differentiation of the diphtheria organism may be a matter of great difficulty. With regard to the rules for practical guidance, however, there is general agreement as to the two following. In the first place, in cases of suspected diphtheria the obtaining of a bacillus in a serum culture from the throat, which has all the morphological and staining characters of the diphtheria bacillus, may be accepted as a positive result for all practical purposes. And, further, most will agree that a similar rule should hold in the first instance with regard to bacilli obtained from the throats of *immediate* contacts. In view, however, of the fact that "diphtheria-like" bacilli without virulence are present in the throats of some healthy individuals, and may also be present along with virulent bacilli in cases of diphtheria, no one should be regarded as a carrier, dangerous to the community, unless the organism in question is proved to possess virulence. Such a rule rests on the assumption that quite avirulent bacilli do not give rise to infection and the results of the accumulated experience of numerous observers support such a view. In the second place, a "diphtheria-like" bacillus obtained from another part of the body, with or without a lesion, should not be accepted as etiologically significant, however closely it resembles the diphtheria bacillus, unless it is found on inoculation to produce the characteristic results. On the other hand, an avirulent organism corresponding fully with the diphtheria bacillus in its morphological, cultural, and fermentative character is meanwhile to be regarded as an avirulent diphtheria bacillus.

Pathogenic Effects of the Diphtheria Bacillus—Summary.—From a study of the morbid changes in diphtheria and of the results produced experimentally by the bacillus and its toxins, the following summary may be given of its action in the body. Locally, the bacillus produces inflammatory change with fibrinous exudation, but at the same time cellular necrosis is also an outstanding feature. Though false membranes have not been produced by the toxins, a necrotic action may result when these are injected subcutaneously. The toxins act

upon the blood vessels, and hence œdema and tendency to hæmorrhage are produced; this action on the vessels is also exemplified by the general congestion of organs. The hyaline change in the walls of arterioles and capillaries, so often met with in diphtheria, is another example of the action of the toxin. The toxins have also a damaging effect on highly developed cells and on nerve fibres. Thus in the kidney cloudy swelling occurs, which may be followed by actual necrosis of the secreting cells, and along with these changes albuminuria is present. The action is also well seen in the case of the muscle fibres of the heart, which may undergo a sort of hyaline change, followed by granular disintegration and associated with leucocytic infiltration. These changes are of great importance in relation to heart failure in the disease. Changes of a somewhat similar nature have been observed in the nerve cells of the central nervous system, those lying near the capillaries, it is said, being affected first. There is also a striking change in the peripheral nerves, which is shown first by the disintegration of the medullary sheaths and to which paralysis is due.

Methods of Diagnosis.—(*a*) *Microscopical Examination*.—Diagnosis by direct microscopic examination is now little used, but it is sometimes justified in cases of urgency, though only in the hands of an experienced observer. In some cases the bacilli are present in characteristic form in such numbers as to leave no doubt in the matter. For the purpose, films are made from a throat swab, or preferably a piece of membrane if obtainable, and stained with methylene-blue and by Neisser's method.

(*b*) *Cultivation*.—The usual routine method is to make a culture from a throat swab on a Löffler's serum slope. No antiseptics should have been applied to the throat for several hours prior to swabbing. If well-defined diphtheritic patches are visible the swab should be rubbed over the affected areas, so as to detach if possible some of the actual exudate; otherwise it should be thoroughly applied to the tonsils and pharynx in such a way as to soak it in the secretion. Where diphtheritic membrane is present the most satisfactory method is to pick off a fragment with forceps and use it for inoculating the culture medium, but in general, swabs if properly used yield satisfactory results in cases of diphtheria. It is undesirable that much time should elapse between the taking of the swab and the making of cultures, and the drying of the swab owing to delay in transmission to a laboratory may be prejudicial to a diagnostic result. The swab is introduced into the culture tube, moistened in the condensation fluid and then smeared over the surface of the serum, being rotated several times in the process. The culture is incubated at 37° C. and examined after eighteen to twenty-four hours. In urgent cases an earlier examination may be carried out, *e.g.* after six to twelve hours. A representative sample of the whole growth is obtained by rubbing a platinum loop over the surface; films are made from this, stained, and examined

in the usual way, Neisser's stain being also applied. Sometimes, when the result is negative, a further period of incubation for twenty-four hours leads to the organisms being found. In clinically suspicious cases a single negative result should not be accepted, but further cultures should be made.

The presence of organisms with the characteristic microscopic appearances and staining reactions in a case presenting the clinical features of diphtheria, may be taken as a positive result. It must be remembered that failure to find the bacilli does not necessarily exclude diphtheria and that their presence does not in all cases connote "clinical diphtheria." In the case of an organism obtained from a suspected carrier or from lesions in parts other than the fauces or larynx, the organism ought to be isolated and further tests applied. Pure cultures may be obtained by means of successive strokes on tellurite media, *e.g.* Horgan and Marshall's or McLeod's medium (p. 75), which are examined after twenty-four hours' incubation at 37° C. The presence of sodium tellurite in culture media greatly facilitates the isolation of the diphtheria bacillus as this substance inhibits the growth of various other organisms which may be derived from the throat (p. 73). The diphtheria bacillus also reduces tellurite and the colonies assume a black or greyish-black appearance which assists in their recognition. (Plates of tellurite media have been used for the direct cultivation of the organism, but on such media the organisms may not show their characteristic staining reactions. This difficulty can be overcome by making cultures in parallel on a plate of tellurite medium and on Löffler's serum; when the plate shows characteristic colonies, films can then be made from the serum culture to demonstrate the staining reactions of the organism and so confirm the diagnosis.) When the organism has been isolated its fermentative properites should be determined. If they correspond with those of *B. diphtheriæ* (production of acid from glucose, no acid from saccharose), the virulence test must be applied; if they do not correspond it may be concluded that the organism is not *B. diphtheriæ*. On McLeod's medium the colony form can be determined: *gravis*, *mitis*, or *intermediate*; and the biological type can be identified further by the starch fermentation test.

(*c*) *Virulence Test.—Subcutaneous method.*—For this purpose the bacillus in question should be grown on a serum slope; the whole of the growth is scraped off and suspended in saline and then injected subcutaneously into a guinea-pig of 250 to 300 grams weight. If the organism is *B. diphtheriæ* the animal will die with the characteristic appearances (p. 383). It is advisable, by way of control, to make at the same time a similar injection into a guinea-pig which has been protected by an intraperitoneal injection, on the previous day, of 500 units of diphtheria antitoxin. The protected animal should show no signs of illness. The *intracutaneous method* (p. 162) is now generally used, as a number of tests can be made on the same animal. The procedure is as follows. Two normal white guinea-pigs of 400 grams weight are selected; one of them is passively immunised on the day before the test by an intraperitoneal injection of 1000 units of diphtheria antitoxin. They are depilated on the abdomen, then an intracutaneous injection is made into each animal of 0·2 c.c. of a suspension of a pure eighteen to twenty-four hours' culture on Löffler's serum which has been

diluted till its opacity corresponds to about 500 million organisms per c.c. In this way a series of cultures may be tested on one pair of animals, but the injections should be, at least, 1 inch apart. About six hours after the injections the unprotected animal should receive a "following dose" of 125 units antitoxin intraperitoneally in order to prevent its death from toxæmia due to virulent cultures; this dose of antitoxin does not interfere with the development of reactions. The results are best read after forty-eight to seventy-two hours. A virulent culture causes at the site of injection a small erythematous patch about 15 mm. in diameter which, at the time of reading, has advanced to superficial necrosis. Avirulent organisms produce little or no reaction. In the immunised animal a virulent organism produces practically no reaction or, at most, a raised red papule which fades rapidly on the second or third day. In cases where it is difficult to obtain a pure culture, what is called the "immediate" or "crude" virulence test may be applied. In this case the whole of an impure serum culture containing the organism may be injected subcutaneously, or a portion may be used by the intracutaneous method. As death may be produced by other organisms if present, care must be taken to ascertain that the characteristic effects of *B. diphtheriæ* are produced, and it is essential to use another animal treated with antitoxin as a control.

Folger serum-swab method for rapid bacteriological diagnosis of diphtheria.—This has been found of value where a bacteriological diagnosis is required within four hours. An ordinary throat swab is dipped in undiluted unheated sterile horse or ox serum (which must, of course, be free from any preservative). The excess of serum is then squeezed out against the side of the tube and the remaining serum is coagulated by heating at 80° C. for fifteen minutes in a serum inspissator or oven or roughly by holding the moist swab momentarily in the flame of a bunsen or spirit lamp. The serum-swab is applied to the throat so that the side of the swab is rubbed over the affected area. The swab is then incubated and from it films are made after two to four hours and stained in the usual way. It is claimed that after two hours positive results can be obtained in 80 per cent. of diphtheria cases and in 95 per cent. after four hours. After incubation for four hours the swab can be used for the inoculation of a plate of tellurite medium if further investigation is required or if the organism is to be isolated for the virulence or other detailed tests (Parish).

Hofmann's Bacillus.—(*Corynebacterium hofmanni*).—This organism, described by Hofmann in 1888, is probably the same as one observed by Löffler in the previous year, and regarded by him as being a distinct species from the diphtheria bacillus. The organism is a shorter bacillus than the diphtheria bacillus, with usually a single unstained area running across it, though sometimes there may be more than one (Fig. 66). The typical beaded appearance is rarely seen, and the characteristic reaction with Neisser's stain is not given, though in old cultures a few granules which stain deeply may sometimes be found. Involution forms may sometimes be produced by it. It grows readily on the same media as the diphtheria bacillus, but the colonies are whiter and more opaque. It does not form acid from glucose or other sugars, and is non-pathogenic to the guinea-pig. It is usually a relatively easy matter to distinguish this organism from the diphtheria bacillus.

Hofmann's bacillus is of comparatively common occurrence in the throat in normal as well as diseased conditions, including diphtheria; it seems to be specially frequent in under-nourished children of the poorer classes. Cobbet found it 157 times in an examination of 692 persons, of whom 650 were not suffering from diphtheria. According to Boycott's statistics the time of its maximum seasonal prevalence precedes that of the diphtheria bacillus. Knowles, on the other hand, in India, found a greatly increased frequence of Hofmann's bacillus during an epidemic of diphtheria, whereas at other times it was relatively rare. There is no evidence that Hofmann's bacillus is pathogenic nor can it be transformed into a virulent *B. diphtheriæ* or *vice versa*, though this was at one time maintained by some.

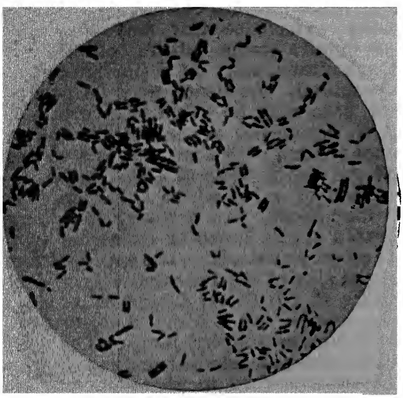


FIG. 66.—Pseudo-diphtheria bacillus (Hofmann's). Young agar culture. See also Plate III., Fig. 15.
Stained with thionin. ×1000.

Xerosis Bacillus (*Corynebacterium xerosis*).—This term has been given to an organism first observed by Kuschbert and Neisser in xerosis of the conjunctiva. It, however, has since been found in many other affections of the conjunctiva and also in normal conditions; in fact, it is usually commensal. Morphologically it is practically similar to the diphtheria bacillus, and even in cultures presents very minor differences; it, however, grows more slowly on serum, and its colonies are small and scaly, have a tough consistence, and a somewhat irregular margin. It is non-virulent to animals and produces acid from glucose and also from saccharose. The last property distinguishes it from the diphtheria bacillus. It is still doubtful whether it has any effect in the human subject, though it increases in numbers in abnormal states of the conjunctiva. Its morphological characters are shown in Fig. 67.

Bacillus Pseudo-tuberculosis Ovis—Preisz-Nocard Bacillus (*Corynebacterium ovis*).—This type of organism is responsible for caseous lymphadenitis of sheep and cattle and ulcerative lymphangitis of horses (*pseudo-farcy*). A similar organism has also been found in a pseudo-tuberculous disease of mice. In morphology little or no distinction can be drawn from the diphtheria bacillus. The colonies on a serum medium tend to show concentric markings, and often develop yellow pigmentation. The growth is rather friable. Gelatin is liquefied, and glucose, maltose and dextrin are fermented but not lactose, saccharose, or mannitol. A hæmolysin which lyses rabbit and horse erythrocytes is produced. Like the diphtheria bacillus this organism produces an

exotoxin, but it differs from that of the former. According to Petrie and McClean this toxin when injected into the skin of a guinea-pig produces a papular lesion which may become pustular in character. Injected subcutaneously it produces death, and its action is not neutralised by diphtheria antitoxin; there is no congestion of the suprarenals and no pleural exudate.

Bacillus Pyogenes (*Corynebacterium pyogenes*).—This organism produces suppurative lesions in pigs, *e.g.* liver abscesses, arthritis, and also in cattle (*e.g.* mastitis). It is diphtheroid in its morphological characters, but is usually very short (2 μ) and sometimes almost coccal. While it may show irregular staining of the protoplasm, metachromatic granules are absent. The colonies on coagulated serum produce small pits of liquefaction, and gelatin is also liquefied. A hæmolysin is produced in culture. Glucose, galactose, lactose, maltose are fermented, but not mannitol. Statements regarding the fermentation of saccharose have differed. Rabbits are susceptible to inoculation, intravenous injection producing a pyæmic condition. Guinea-pigs and mice are less susceptible.

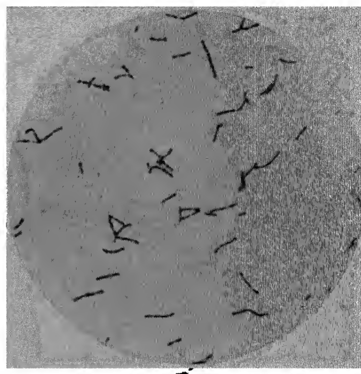


FIG. 67.—Xerosis bacillus from a young agar culture. Stained with methylene-blue. ×1000.

Two other diphtheroid organisms have been described in animal diseases: *Corynebacterium renale* and *Corynebacterium equi* (see Merchant). *C. renale* is associated with pyelonephritis of cattle. It differs from the other species described above in the absence of liquefaction of gelatin and coagulated serum. In morphology it is typically diphtheroid with metachromatic granules. On serum medium it produces moist colonies with yellowish pigmentation. Glucose and lævulose are fermented, but not the other carbohydrates which are generally used for fermentation tests. *C. equi* has been described in pneumonia of colts. It is characterised by its profuse viscid growth and red pigmentation. Carbohydrates are not fermented.

BACTERIUM MONOCYTOGENES (*Listerella monocytogenes*)

This organism was described by Murray, Webb and Swann in a disease of rabbits in which an excess of large mononuclear cells in the blood was a striking feature. Similar organisms have also been reported in various animals and in meningeal infections in man (Burn). The organism when inoculated in rabbits and guinea-pigs, produces a monocytosis like that of the natural infection and may localise in the myocardium and liver, producing necrotic lesions and

in the meninges with associated inflammation. The microscopical characters are those of a Gram-positive, non-sporing bacillus with rounded ends, varying in length from 1 to 4 μ and about 0·5 μ broad. No branching is observed, but filamentous forms have been seen. The organism is motile and a single terminal flagellum has been described. The staining may be either uniform or irregular, the organism in the latter case having a beaded appearance. Cultures can be obtained under strictly aerobic conditions at 37° C. on ordinary media, but growth is better on a medium containing liver extract. The colonies are small and transparent. Glucose is fermented and to a slight extent various other carbohydrates. Indole is not produced. Gelatin is not liquefied. The biological relationships of this type of organism are doubtful. Some observers have placed it in a separate genus (*Listerella*), but it may be related either to the *Erysipelothrix* or diphtheroid group.

CHAPTER X

THE TUBERCLE BACILLUS.

THE cause of tuberculosis was proved by Koch in 1882 to be the organism now known as the tubercle bacillus. Probably no other single discovery has had a more important effect on medical science. It has supplied infallible methods for determining the tuberculous nature of lesions, and has also given the means of studying the modes and paths of infection.

Historical.—By the work of Villemin and of Cohnheim and Salomonsen (1870–80) it had been demonstrated that tuberculosis was an infective disease. The latter observers found on inoculation of the anterior chamber of the eye of rabbits with tuberculous material, that in many cases the results of irritation soon disappeared, but that after a period of incubation, usually about twenty-five days, small tuberculous nodules appeared in the iris; afterwards the disease gradually spread, leading to disorganisation of the globe of the eye. Later still, the lymphatic glands became involved, and finally the animal died of acute tuberculosis. The question remained as to the nature of the virus, the specific character of which was thus established, and this question was answered by the work of Koch, which will remain as a classical masterpiece of bacteriological research, both on account of the great difficulties which he successfully overcame and the completeness with which he demonstrated the relations of the organism to the disease. The two chief difficulties were, first, the demonstration of the bacilli in the tissues, and, secondly, the cultivation of the organism outside the body. The tubercle bacillus cannot be demonstrated by a simple watery solution of a basic aniline dye, and it was only after staining for twenty-four hours, with a solution of methylene-blue with caustic potash added, that he was able to reveal the presence of the organism. All attempts to cultivate it on the ordinary media failed, and he succeeded in obtaining growth only on solidified blood serum, the method of preparing which he himself devised, inoculations being made on this medium from the organs of animals artificially rendered tuberculous. The fact that growth did not appear till the tenth day at the earliest, might easily have led to the hasty conclusion that no growth took place. The organism was cultivated by the above method from a great variety of sources, and by a large series of inoculation experiments on various animals, performed by different methods, Koch conclusively proved that bacilli from these different sources produced the same tuberculous

lesions and were really of the same species. His work demonstrated that such conditions as lupus, "white swelling" of joints, scrofulous disease of glands, etc., were really tuberculous in nature.

Tuberculosis in Animals.—Tuberculosis is not only the most widely spread of all infective diseases affecting the human subject, and one of the chief causes of death, but there is probably no other disease which affects the domestic animals so widely. We need not here describe in detail the various tuberculous lesions in the human subject, but some facts regarding the disease in the lower animals may be given, as this subject is of great importance in relation to the infection of the human subject.

Amongst the domestic animals the disease is commonest in cattle (bovine tuberculosis), in which animals the lesions are very varied, both in character and distribution. In most cases the lungs are affected, and contain numerous rounded nodules, many being of considerable size; these may be softened in the centre, but are usually of fairly firm consistence and may be calcified. There may be in addition caseous pneumonia, and also small tuberculous granulations. Along with these changes in the lungs, the pleuræ are often affected, and show numerous nodules, some of which may be of large size, firm and pedunculated, the condition being known in Germany as *Perlsucht*, in France as *pommelière*. Lesions similar to the last may be chiefly confined to the peritoneum and pleuræ. In other cases, again, the abdominal organs are principally involved The udder becomes affected in a certain proportion of cases of tuberculosis in cows, but primary affection of this gland is very rare. Tuberculosis is also a comparatively common disease in pigs, in which animals it affects in many cases the abdominal organs, in other cases produces a sort of caseous pneumonia, and sometimes is met with as a chronic disease of the lymphatic glands, the so-called "scrofula" of pigs. Tuberculous lesions in the muscles are less rare in pigs than in most other animals. In the horse the abdominal organs are usually the primary seat of the disease, the spleen being often enormously enlarged and crowded with nodules; sometimes, however, the primary lesions are pulmonary. In sheep and goats tuberculosis is of rare occurrence, especially in the former animals. It may occur spontaneously in dogs, cats, and in the large carnivora. It is also met with in monkeys in confinement, and leads to a very rapid and widespread affection, the nodules having a special tendency to soften and break down into a pus-like fluid. Guinea-pigs and rabbits may acquire the infection when kept in the vicinity of animals with open tuberculous lesions.

Tuberculosis in fowls (avian tuberculosis) is a common and very infectious disease, nearly all the birds in a poultry-yard being sometimes affected.

The disease in animals thus presents great variations in character, and may differ in many respects from that met with in the human subject. The relations of the different forms of tuberculosis are discussed below, but it may be stated here that

two chief types of tubercle bacilli in mammals are now recognised—*a human type* which is the common cause of tuberculosis in the human subject, and a *bovine type* which produces bovine tuberculosis and also a certain proportion of cases of human tuberculosis.

Tubercle Bacillus (Human Type) (*Mycobacterium tuberculosis hominis*)—**Microscopical Characters.**—Tubercle bacilli are rod-shaped organisms which usually measure 2·5 to 3·5 μ in length, and 0·3 μ in thickness, *i.e.* in proportion to their length they are comparatively thin (Figs. 68 and 69). Sometimes, however, longer forms, up to 5 μ or more in length, are met with, both in cultures and in the tissues. They are straight or slightly curved, and are of uniform thickness, or may show slight swelling at their extremities. When stained they appear uniformly coloured, or may present small uncoloured spots along their course, with darkly stained parts between. There is no evidence that such appearances represent spore formation, and it has been shown that "beaded" bacilli have no higher powers of resistance than those which stain uniformly.

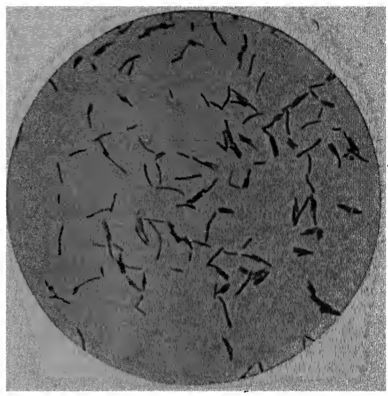


Fig. 68.—Tubercle bacilli of the human type, from a pure culture on glycerol agar.
Stained with carbol-fuchsin. ×1000.

The bacilli in the tissues occur scattered irregularly or in little masses. They are usually single, or two are attached end to end and often form in such a case an obtuse angle. True chains are not formed, but occasionally short filaments are met with. In cultures the bacilli form masses in which the rods are closely applied to one another and arranged in a more or less parallel manner. Tubercle bacilli are devoid of motility.

Staining Reactions.—The tubercle bacillus takes up the ordinary stains very slowly and faintly, and for successful staining one of the most powerful solutions ought to be employed, *e.g.* crystal violet or fuchsin, along with aniline water or carbolic acid. Further, such solutions require to be applied for a long time, or the staining must be accelerated by heat, the

solution being warmed till steam arises and the specimen allowed to remain in the hot stain for some minutes. One of the best and most convenient methods is that of Ziehl-Neelsen (see p. 120). The bacilli present this further peculiarity, discovered by Ehrlich, that after staining has taken place they resist decolorising by solutions which readily remove the colour from the tissues and from other organisms which may be present. Such decolorising agents are sulphuric or nitric acid in 20 per cent. solution. Preparations can thus be obtained in which the tubercle bacilli alone are coloured by the stain first used, and

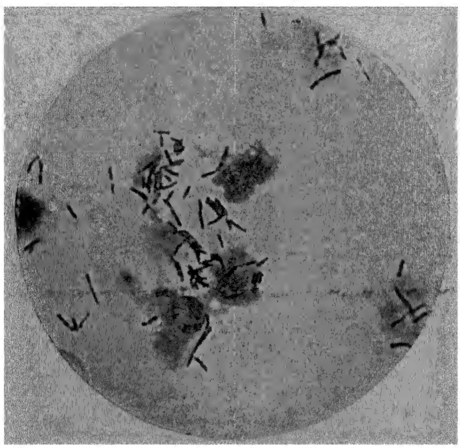


Fig. 69.—Tubercle bacilli in phthisical sputum; they are longer than is often the case. See also Plate II., Fig. 7. Film preparation, stained with carbol-fuchsin and methylene-blue. ×1000.

the tissues can then be coloured by a contrast stain. The tubercle bacillus when stained as above described will resist decolorising by 20 per cent. acid for twenty-four hours. Other bacilli have been discovered which present the same staining reactions as tubercle bacilli; they are therefore called "acid-fast" (*vide infra*). (The spores of many bacilli become decolorised more readily than tubercle bacilli, though some retain the colour with equal tenacity.) The acid-fast bacilli have been included in the group *Mycobacterium*. The tubercle bacillus is Gram-positive, but is not readily stained by the usual Gram's method. Hot water quickly decolorises stained organisms of the acid-fast group, including the tubercle bacillus.

Aberrant Forms.—Though such are the characters of the organism as usually met with, other appearances are sometimes found. In old cultures, for example, very much larger elements may occur. These may be in the form of long filaments, sometimes swollen or clubbed at their extremities, may be irregularly beaded, and may even show the appearance of branching. Their significance has been variously interpreted, for while some look upon them as degenerate or involution forms, others regard them as indicating a special phase of the organism, allying it with the higher bacteria. It has also been found that tubercle bacilli in the tissues may produce

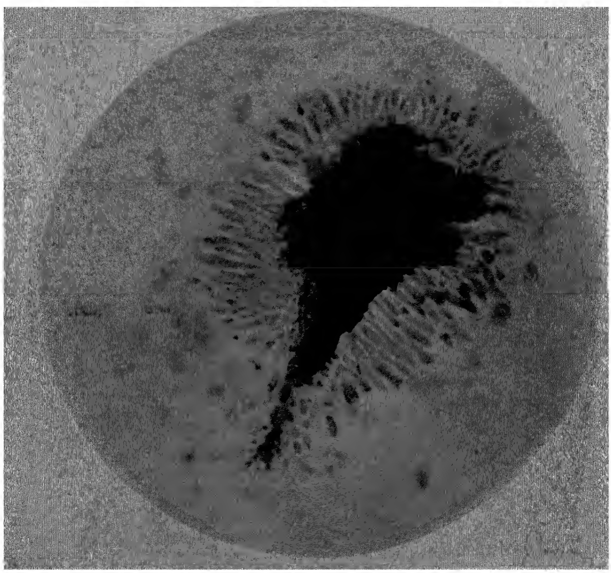


FIG. 70.—Large clump of tubercle bacilli (bovine type) in lung of rabbit (experimentally inoculated) showing club formations. ×1000.

radiating structures with club-like forms at the periphery closely similar to those of actinomyces. This was found by Babés and also by Lubarsch to be the case when the bacilli were injected under the dura mater and directly into certain solid organs, such as the kidneys in the rabbit. We have observed this appearance in the lungs of rabbits inoculated intravenously with a large dose of the bovine type of tubercle bacillus (Fig. 70). Similar results obtained with other acid-fast bacilli will be mentioned below, and these organisms would appear to form a group closely allied to the *Streptothrices*, the bacillary parasitic form being one stage of the life-history of the organism.

Granular Forms.—Much maintained that the tubercle bacillus

may exist in a form which is not acid-fast and also in the form of free granules demonstrable by his modification of Gram's method. There seems to be no doubt that in certain conditions more tubercle bacilli can be demonstrated in the tissues by Much's method than by the Ziehl-Neelsen method. Sometimes tubercle bacilli can be stained by Gram's method employed in the usual way.

According to Karwacki cultures of the granular phase can be obtained under certain conditions from pathological material and from typical acid-fast bacilli, *e.g.* from a culture one to three years old ; the colonies are dew-drop-like and may be pigmented (yellow or orange). Pathogenic effects are variable, but sometimes caseous tubercles have resulted in inoculated animals, and acid-fast bacilli have been detected in some of the lesions.

Cyanophile Bacilli.—This term has been applied to elements which are decolorised in the Ziehl-Neelsen method and are counter-stained with methylene-blue. It is stated that if typical cultures of the tubercle bacillus on glycerol-agar are kept at room temperature for several months, subculture may yield a growth of non-acid-fast bacilli, the colonies being small and sometimes pigmented. The bacilli tend to resemble diphtheroid organisms Tubercle-like lesions have been produced with these forms but typical tuberculosis does not result. On transfer to a rich medium, acid-fast bacilli are developed (Karwacki)

Filter-passing Forms of the Tubercle Bacillus.—It has been claimed that tuberculous products and cultures of tubercle bacilli may contain the organism in a form which passes through bacterial filters. Rarely, in cultures from such filtrates organisms have grown which were non-acid-fast but later became acid-fast. On inoculation of the filter-passing form into guinea-pigs there develops merely an enlargement of lymphatic glands and spleen ; acid-fast bacilli may be present or they may not be found but may appear after repeated passage in fresh animals, in which also the infection becomes more virulent. According to Nègre and Valtis filterable forms when inoculated into guinea-pigs become converted to the acid-fast stage *in vivo* by injecting an acetone extract of tubercle bacilli. The lymph glands in the inoculated area then show typical bacilli and subinoculation with the affected gland and injection of acetone extract into another animal produces generalised tuberculosis. According to Calmette and his co-workers, the filterable form when inoculated into a pregnant guinea-pig is able to pass through the placenta and develop into the usual acid-fast bacilli in the fœtus. Further, the filterable form has been stated to occur in the organs of infants and fœtuses of tuberculous mothers. Although confirmatory results have been obtained by a number of workers, others have failed entirely to obtain cultures or to infect animals with filtrates (Gloyne, Glover and Griffith). It is possible that the passage of a few bacilli through a filter might account for the results which have been claimed in regard to the supposed filterable phase. Accordingly, the claims regarding such phases of the tubercle bacillus require fuller substantiation.

Chemical Composition.—When tubercle bacilli are extracted with organic solvents such as alcohol or ether, especially with the aid of heat, substances are dissolved out which show the acid-fast property. But it is extremely difficult to deprive the bacilli of their characteristic staining reaction by such treatment. Aronson found

that extraction with a boiling mixture of hydrochloric acid and alcohol removed the acid-fast property, and Bulloch and McLeod by a similar process extracted an acid-fast wax. Long has shown that if tubercle bacilli are extracted as completely as possible with alcohol and petroleum ether and are then treated with N/1 HCl a subsequent extraction with the organic solvents removes a further quantity of "firmly bound" lipoid substances and destroys the acid-fastness and integrity of the bacilli, both of which had resisted the primary extraction. But *B. subtilis* contains practically as much of the firmly bound lipoid although, like other non-acid-fast organisms, its total content in substances soluble in organic solvents is much less than that of tubercle bacilli. Browning and Gulbransen have found that films of tubercle bacilli are rapidly deprived of acid-fastness by treatment with a mixture of alcohol and chloroform containing a minute amount of HCl, *e g.* N/100, whereas in the absence of the acid several months' treatment leaves the bacilli still acid-fast.

The facts support Long's view that it is the manner of distribution of the waxy or lipoid substance in the bodies of the bacilli which confers the typical staining properties on tubercle bacilli. This accords also with Koch's observation that the bacilli when disintegrated by trituration cease to be acid-fast. Bacilli which have lost their acid-fast properties by the above methods are still stainable by basic aniline dyes, though they take up the stain feebly. Laidlaw and Dudley have obtained glycogen from tubercle bacilli and also a carbohydrate complex of the nature of a gum. The latter gives specific precipitation with an immune serum.

More recently, isolated chemical constituents of the tubercle bacillus have been studied by Sabin and others, with specially interesting results as regards the cellular reactions produced by their injection. A saturated hexacosanic acid, "phthioic acid," has been separated and also an unsaponifiable wax, the latter of which gives the acid-fast reaction. Phthioic acid on injection excites the formation of tubercles, *i.e.* proliferation of endothelioid cells with the accumulation of mononuclear cells, while the wax stimulates the formation of connective tissue. Polysaccharide fractions have also been separated and have been found to be toxic to the neutrophile leucocytes. These findings throw some light on the tissue changes observed in tuberculous lesions.

Cultivation.—The medium first used by Koch was inspissated blood serum (*vide* p. 67). If inoculations are made on this medium with tuberculous material free from other organisms, there appear in from ten to fourteen days minute points of growth of dull whitish colour, rather irregular, and slightly raised above the surface. Koch compared the appearance of these to that of small dry scales. In such cultures the growths usually reach only a comparatively small size and remain separate, becoming confluent only when many occur close together. In subcultures, however, growth is more luxuriant and may come to form a dull wrinkled film of

whitish colour, which may cover the greater part of the surface of the serum and at the bottom of the tube may grow over the surface of the condensation water on to the glass (Fig. 71). The growth is always of a dull appearance, and has a considerable degree of consistence, so that it is difficult to dissociate a portion thoroughly in a drop of water. In older cultures, when the medium consists of well-pigmented ox serum, the growth may acquire a yellow or orange colour. When the small colonies are examined under a low power of the microscope, they are seen to be extending at the periphery in the form of wavy or sinuous streaks which radiate outward, and which have been compared to the flourishes of a pen. The central part shows similar markings closely interwoven. These streaks are composed of masses of the bacilli arranged in a more or less parallel manner.

A B C

FIG. 71.—Cultures of tubercle bacilli on glycerol agar.

A and B. Mammalian tubercle bacilli of human type: A is an old culture, B one of a few weeks' growth.
C. Aviant ubercle bacillus The growth is whiter and smoother on the surface than the others.

On *egg medium* and especially on *glycerol egg medium* the organism grows well, producing an abundant irregular, wrinkled or mammillated layer which has usually a yellowish, buff, or pinkish colour. These media, particularly when made up with digest broth, are specially suitable for direct cultivation from the tissues.

On *glycerol agar*, which was first introduced by Nocard and Roux as a medium for the culture of the tubercle bacillus, growth takes place in subcultures, but this medium is not suitable for obtaining primary cultures from the tissues, inoculations with tuberculous material usually yielding a nega-

tive result. The growth has practically the same characters as on serum. The organism also flourishes well on *glycerol potato*, and this medium is suitable for primary cultures from tuberculous lesions. In *glycerol broth*, especially in a shallow layer in a flask, tubercle bacilli grow readily in the form of little white masses, which fall to the bottom and form a powdery layer. If, however, the growth be started on the surface, it spreads superficially as a dull whitish wrinkled pellicle which may reach the wall of the flask; this mode of growth is specially suitable for the production of tuberculin (*vide infra*). The culture has a characteristic fruity odour. On ordinary agar and on gelatin media no growth takes place. The use of animal tissues in glycerol broth as a medium for the growth of the tubercle bacillus was introduced by Frugoni, and is one which gives excellent results. He recommends that small wedges of rabbit's lung should be sterilised in the autoclave, and placed in tubes of glycerol broth in such a way that their surface is kept moist by the medium, without the fragments being submerged. The growth is probably more rapid and luxuriant than in any other method. Since tubercle bacilli grow best with free access to the oxygen of the air, cultures which have been sealed to prevent evaporation should be opened from time to time.

The optimum temperature for growth is 37° to 38° C. Growth ceases about 42° and usually below 28°, but on long-continued cultivation outside the body and in special circumstances growth may take place at a lower temperature. Cultures may remain alive for long periods either at room temperature or at 37° C., even for several years. Some strains retain their virulence after cultivation for many years, others soon lose virulence.

Dissociation.—As first shown by Petroff and his co-workers strains of tubercle bacilli may be dissociated into variants which differ in colony characteristics. These have been designated "smooth" (S), "rough" (R) and "intermediate" according to the appearances of colonies, but it is doubtful whether such variations are strictly analogous to those similarly designated in other bacterial groups. Generally, the colonies of the S type are round with an almost entire edge, either relatively flat or convex and dome shaped, smooth and glistening, soft or slimy in consistence and easily emulsified (Fig. 72). The R type colonies are irregular, raised or heaped up and sometimes crater-like, rough and dull in appearance, friable and difficult to emulsify, forming an unstable suspension in physiological saline (Fig. 73). Recently Steenken, Oatway and Petroff have dissociated a virulent strain of the human type into stable "smooth" and "rough" forms: the former consisted of flat wrinkled or stippled cream-coloured colonies with spreading veil-like peripheries, the latter of discrete, slightly chromo-

genic, dry, raised, crater-like colonies which had a clear-cut periphery; the "rough" type was avirulent, while the "smooth" form was actively tuberculogenic. It seems possible, however, that this "smooth" type is in reality an intermediate form between the true S and R Petroff and his co-workers had found previously that an S variant derived from the avirulent culture of Calmette and Guérin (B.C.G.—*vide* p. 438) may be pathogenic.

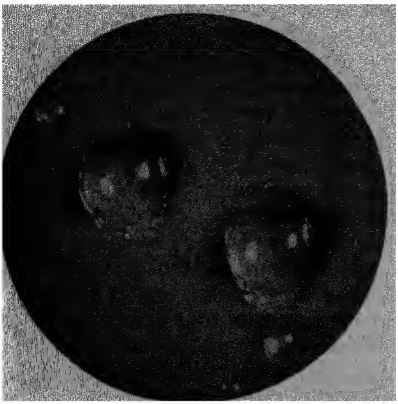


FIG. 72.—Colonies of the S type, bovine tubercle bacillus. 56 days' growth. ×10. From photograph by Dr. M. H. Christison).

Powers of Resistance.—Tubercle bacilli have considerable powers of resistance to external influences, and can retain their vitality for a long time outside the body in various conditions; in fact, in this respect they may be said to occupy an intermediate position between spores and spore-free bacilli. Dried phthisical sputum has been found to contain virulent bacilli after two months, and similar results are obtained when the bacilli are kept in distilled water for several weeks. So also they resist for a long time the action of putrefaction, which is rapidly fatal to many pathogenic organisms. Sputum has been found to contain living tubercle bacilli even after being allowed to putrefy for several weeks (Fraenkel, Baumgarten), and the bacilli have been found to be alive in tuberculous organs which have been buried in the ground for a

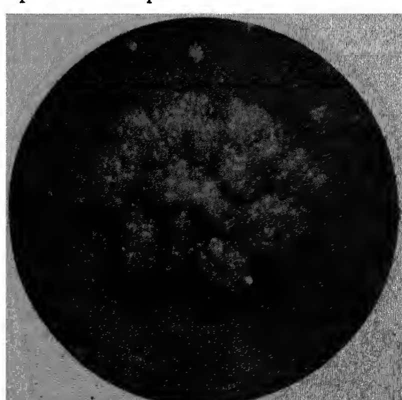


FIG. 73.—Colony of R type, bovine tubercle bacillus. 63 days' growth. ×10. (From photograph by Dr. M. H. Christison)

similar period. They are not killed by being exposed to the action of the gastric juice for six hours; they will survive a temperature below freezing point for many months. It has been found that when completely dried they can resist a temperature of 100° C. for an hour, but, on the other hand, exposure in the moist condition to 60° C. for the same time is usually fatal. It may be stated that raising the temperature to 100° C. kills the bacilli in fluids and in tissues, but in the case of large masses of tissue care must be taken that this temperature is reached throughout. They are killed in less than a minute by exposure to 5 per cent. carbolic acid, and both Koch and Straus found that they are rapidly killed by being exposed to the action of direct sunlight. But it is much more difficult to sterilise sputum containing tubercle bacilli by means of antiseptics, as the physical and chemical characters of the sputum prevent effective penetration of the chemicals used. According to Maddock, tubercle bacilli may survive for six months on soil and manure, while on growing grass they may be found alive and virulent for forty-nine days during ordinary summer weather. These findings are of significance in relation to the infection of pastures by tuberculous cattle.

Action on the Tissues.—The *local lesion* produced by the tubercle bacillus is the well-known tubercle nodule, the structure of which varies in different situations and according to the intensity of the action of the bacilli. After the bacilli gain entrance to a connective tissue such as that of the iris, their first action appears to be on the endothelial and connective-tissue cells, which become somewhat swollen and undergo mitotic division, the resulting cells being distinguishable by their large size and pale nuclei—the so-called endothelioid cells. These proliferative changes may be well seen on the fifth day after inoculation or even earlier. A small focus of proliferated cells is thus formed in the neighbourhood of the bacilli, and about the same time numbers of leucocytes—chiefly lymphocytes—begin to appear at the periphery and gradually become more numerous. Soon, however, the toxic action of the bacilli comes into prominence. The endothelioid cells become swollen and somewhat hyaline, their outlines become indistinct, while their nuclei stain faintly, and ultimately lose the power of staining. The cells in the centre, thus altered, gradually become fused into a homogeneous substance, and this afterwards becomes somewhat granular in appearance. If the central necrosis does not take place quickly, then giant-cell formation may occur in the centre of the follicle, this constituting one of the characteristic features

of the tuberculous lesion; or after the occurrence of caseation giant-cells may be formed in the cellular tissue around. The centre of a giant-cell often shows signs of degeneration, such as hyaline change and vacuolation, or it may be more granular than the rest of the cell. The exact mode of formation of a tubercle follicle varies, however, in different tissues.

There can be no doubt that the cell necrosis and subsequent caseation depend upon the products of the bacilli, and are not due to the fact that the tubercle nodule is non-vascular. This non-vascularity itself is to be explained by the circumstance that young capillaries cannot grow into a part where tubercle bacilli are active, and that the already existing capillaries become thrombosed, owing to the action of the bacillary products on their walls, and ultimately disappear. At the periphery of tuberculous lesions there may be considerable vascularity and new formation of capillaries; also, capillaries may be abundant in forms of tuberculous granuloma in which there is little or no caseation.

Reference has been made above (p. 411) to the tissue reactions produced by different chemical fractions of the tubercle bacillus.

The *general symptoms of tuberculosis*—pyrexia, wasting, etc.—are to be ascribed to the absorption and distribution throughout the system of the toxic products of the bacilli; in the case of phthisical cavities and like conditions where other bacteria are present, the toxins of the latter also play an important part. The occurrence of amyloid change in the organs is believed by some to be chiefly due to the products of other, especially pyogenic, organisms, secondarily present in the tuberculous lesions.

Presence and Distribution of the Bacilli.—A few facts may be stated regarding the presence of bacilli, and the numbers in which they are likely to be found in tuberculous lesions. They are usually very few in number in chronic lesions, whether these are tubercle nodules with much connective-tissue formation or old caseous collections. In caseous material one can sometimes see a few bacilli faintly stained, along with very minute unequally stained granular points; but not infrequently none can be detected. The important fact, however, has been established, that tuberculous material in which no bacilli can be found microscopically may be proved, on experimental inoculation into animals, to be still virulent. In subacute lesions, with well-formed tubercle follicles and little caseation, the bacilli are generally scanty. They are most numerous in acute lesions, especially where caseation is rapidly spreading, for example, in such conditions as caseous catarrhal pneumonia (Fig. 74), acute tuberculosis of the spleen in children, which is often attended

with a good deal of rapid caseous change, etc.; in such conditions they often form large masses which are easily seen under a low power of the microscope, being specially abundant at the margins of the caseous areas. In acute miliary tuberculosis a few bacilli can generally be found in the centre of the follicles; but here they are often much more scanty than one would expect. The tubercle bacillus is one which not only has comparatively slow growth, but retains its form and staining power for a much

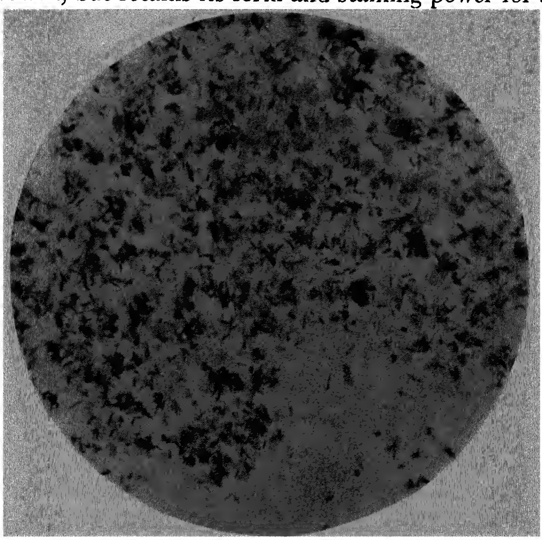


FIG. 74.—Tubercle bacilli in section of human lung in acute phthisis. The bacilli are seen lying singly, and also in large masses. The pale background is formed by caseous material.
Stained with carbol-fuchsin and methylene-blue. ×600.

longer period than most organisms. As a rule, the bacilli are extracellular in position. Occasionally they occur within the giant-cells, also in endothelioid cells and in leucocytes; the intracellular site is, however, commoner in some of the lower animals. In the ox, for example, tubercle bacilli are commonly found within giant-cells, in which they are often arranged in a somewhat radiate manner at the periphery; it is also common to find bacilli in considerable numbers scattered irregularly throughout the cellular connective tissue of the lesions, even when there is little or no caseation present (Fig. 75).

In tuberculosis in the horse and in avian tuberculosis the numbers of bacilli may be enormous, even in lesions which are not specially acute; and considerable variation both in their number and in their site is met with in tuberculosis of other animals.

In discharges from tuberculous lesions which are breaking down, tubercle bacilli are usually to be found. In the sputum of phthisical patients their presence can be demonstrated almost

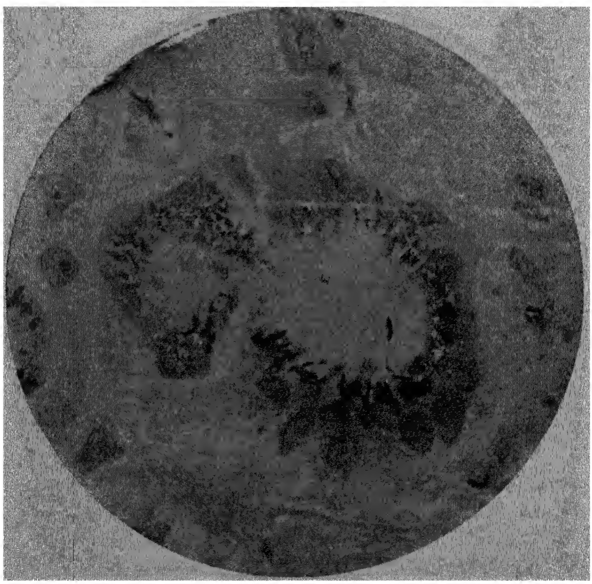


Fig. 75.—Tubercle bacilli in giant-cells, showing the radiate arrangement at the periphery of the cells. Section of tuberculous udder of cow.
Stained with carbol-fuchsin and Bismarck-brown. ×1000.

invariably at some period, and sometimes their numbers are very large. Several examinations may, however, require to be made, also animal inoculation, before any conclusion as to the non-tuberculous nature of a case can be drawn. In tuberculous meningitis the bacilli can often be found in the cerebro-spinal fluid obtained by lumbar puncture, but are frequently scanty. In cases of genito-urinary tuberculosis they are usually present in the urine; but as they are much diluted it is difficult to find them unless a deposit is obtained by means of the centrifuge.

This deposit is examined in the same way as the sputum. The bacilli often occur in little clumps, as shown in Fig. 76. In tuberculous ulceration of the intestine their presence in the fæces may be demonstrated, as was first shown by Koch; but in this case their discovery is usually of little importance as the intestinal lesions, as a rule, occur only in advanced stages when diagnosis is no longer a matter of doubt. It should be noted that cultural methods (*vide* p. 444) may in some cases reveal the presence of tubercle bacilli in pathological material in which they cannot be detected by direct microscopic examination.

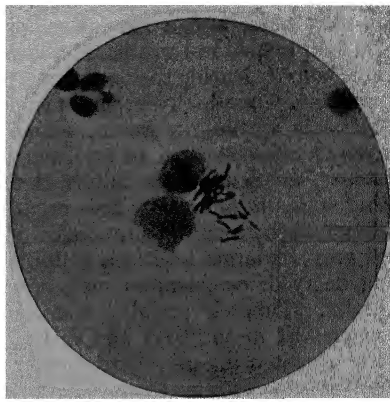


Fig. 76.—Tubercle bacilli in urine; showing one of the characteristic clumps in which they often occur.
Stained with carbol-fuchsin and methylene-blue. ×1000.

Claims have been made that tubercle bacilli can be demonstrated by microscopic or cultural methods in the circulating blood in a large proportion of tuberculous cases (Rosenberger, Löwenstein). Certain of these observations have been possibly fallacious and such findings have not been confirmed.

Experimental Inoculation. — Tuberculosis can be artifically produced in animals in a great many different ways—by injection of the bacilli into the subcutaneous tissue, into the peritoneum, into the anterior chamber of the eye, into the veins; by feeding the animals with the bacilli; and, lastly, by making them inhale the bacilli suspended in the air.

The exact result, of course, varies in different animals and according to the method of inoculation, but we may state generally that when introduced into the tissues of a susceptible animal, the bacilli produce locally the lesions above described, terminating in caseation; that there occurs a tuberculous affection of the neighbouring lymphatic glands, and that lastly there may be a rapid extension of the bacilli to other organs by the blood stream and the production of general tuberculosis. Of the animals generally used for the purpose, the guinea-pig is most susceptible.

When a guinea-pig is inoculated subcutaneously with tubercle bacilli from a culture, or with material containing them, such as phthisical sputum, a local swelling gradually forms which is usually well marked about the tenth day. This swelling becomes softened and caseous, and may break down, leading to the formation of an irregular ulcerated area with caseous lining. The lymphatic glands in relation to the parts can generally be found to be enlarged and of somewhat firm consistence, about the end of the second or third week. Later, in them also caseous change occurs, and a similar condition may spread to other groups of glands in turn, passing also to those on the other side of the body. During the occurrence of these changes, the animal loses weight, gradually becomes cachectic, and ultimately dies, sometimes within six weeks, sometimes not for two or three months. *Post mortem*, in addition to the local and glandular changes, an acute tuberculosis is usually present, the spleen being specially affected. This organ is swollen, and is studded throughout by numerous tubercle nodules, which may be minute and grey, or larger and of a yellowish tint. If death has been long delayed, calcification may have occurred in some of the nodules. Tubercle nodules, though rather less numerous, are also present in the liver and in the lungs, the nodules in the latter organs being usually of smaller size though occasionally in large numbers. The kidneys only occasionally show nodules. The extent of the general infection varies ; sometimes the chronic glandular changes constitute the outstanding feature. Statements as to differences in the pathogenic effects of bacilli from human and bovine sources will be found below.

It has been long recognised that silicosis of the lung predisposes to tuberculosis, and the factors concerned have received considerable study. It was shown by Gye and Kettle that if tubercle bacilli were injected along with silica into the subcutaneous tissues of the mouse, the bacilli proliferated readily in the area of coagulation necrosis produced by the silica and seemed to be protected from the tissue defences. Kettle also found that when tubercle bacilli were injected into the circulation of rabbits and mice, they tended to localise in subcutaneous lesions produced by various agents which brought about increased vascularity and tissue necrosis. When silica was used as the provocative agent this localisation was invariable and there was extensive proliferation of the bacilli in the local lesion. Cummins and Weatherall have confirmed Gye and Kettle's findings, but they point out that the effect of silica is local and transitory.

Action of Dead Tubercle Bacilli.—The interesting fact has been established by independent investigators, that tubercle bacilli in the dead condition, when introduced into the tissues in sufficient numbers, can produce tubercle-like nodules. Prudden and Hoden-

pyl, by intravenous injection in rabbits of cultures sterilised by heat, produced in the lungs small nodules in which giant-cells, but no caseation, were occasionally present, and which were characterised by more growth of fibrous tissue than in ordinary tubercle. The subject was very fully investigated, with confirmatory results, by Straus and Gamaleia, who found that, if the numbers of bacilli introduced into the circulation were large, there resulted very numerous tubercle nodules with well-formed giant-cells, and occasionally traces of caseation. The bacilli can be well recognised in the nodules by the ordinary staining method. Similar nodules can be produced by intraperitoneal injection. Subcutaneous injection, on the other hand, produces a local abscess, but in this case no secondary tubercles are found in the internal organs. Further, in many of the animals inoculated by the various methods, a condition of marasmus sets in and gradually leads to a fatal result. These experiments, which have been confirmed by other observers, show that even after the bacilli are dead they preserve their staining reactions in the tissues for a long time, and also that there are apparently contained in the bodies of the dead bacilli certain substances which act locally, producing proliferative and, to a less extent, degenerative changes, and which also markedly affect the general nutrition. Reference has been made to this question in connection with the chemical constituents of the bacilli (p. 411). It has been found that animals inoculated with dead tubercle bacilli afterwards give the tuberculin reaction (*vide infra*).

Varieties of Tuberculosis.—1. *Human and Bovine Tuberculosis.*—This subject first aroused special interest owing to Koch's address at the Tuberculosis Congress in 1901, in which he stated his conclusion that human and bovine tuberculosis are practically distinct, and that if susceptibility of the human subject to the latter really exists, infection is of very rare occurrence—so rare that it is not necessary to take any measures against it. Previously to this, Theobald Smith had pointed out differences between human and bovine tubercle bacilli, the most striking being that the latter possess a much higher virulence to the guinea-pig, rabbit, and other animals, and in particular that human tubercle bacilli, on inoculation into oxen, produce either no disease or only local lesions without any dissemination. Koch's conclusions were based chiefly on the result of his inoculation of the bovine species with human tubercle bacilli, the result being confirmatory of Smith's, and also on the supposition that infection of the human subject through the intestine is of very rare occurrence.

After Koch's communication an enormous amount of work was done on this subject, and commissions of inquiry were appointed in various countries. We may summarise the chief facts which have been established. Practically all observers are agreed that there are two chief types of tubercle bacilli,

which differ both in their cultural characters and in their virulence—a *bovine type* and a *human type*. The bacillus of the bovine type (*Mycobacterium tuberculosis bovis*), when cultivated, is often shorter and thicker and more regular in size (Fig. 77); whilst its growth on various culture media is scantier than that of the human type. From the latter character the British Royal Commission applied the term *dysgonic* to the bovine and *eugonic* to the human type. For distinguishing the growth characters of the two types, egg media (p. 70) are especially suitable. On this medium the human type produces an abundant, dry, and wrinkled or verrucose growth, which has often a yellowish or pinkish tint; while the bovine type forms a thin whitish layer, smooth or somewhat granular, rather moist in appearance, and the growth is much more easily broken up. The difference between the two types is accentuated by the addition of glycerol to the medium; this greatly favours the growth of the human type, while it does not favour, or even inhibits, the growth of the bovine type. In fact, on glycerol egg medium primary cultures of the latter often fail. On inspissated serum bovine cultures are never pigmented as the former type often is. These differences are most marked in the early cultures; in later subcultures they tend to diminish. The vitality of the bovine type is less on artificial media, cultures having sometimes a tendency to die out. As already stated, there is also a great difference in virulence towards the lower animals, the bacillus from the ox having a much higher virulence. This organism when injected in suitable quantities into the ox produces a local tuberculous lesion, which is usually followed by a generalised and fatal tuberculosis; whereas injection of human tubercle bacilli usually produces no more than a local lesion, which undergoes retrogression. Corresponding differences emerge in the case of the rabbit; in fact, intravenous injection of suitable quantities (*e.g.* 0·01–0·1 mgrm. of young culture suspended in 1 c.c. of saline) in this animal is the readiest method of distinguishing the two types—an acute tuberculosis resulting with the bovine,

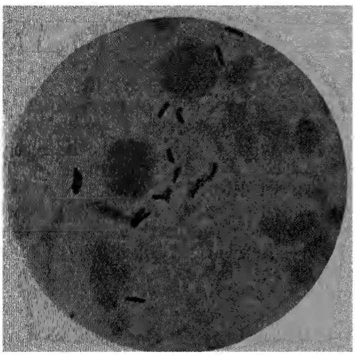


FIG. 77.—Bovine tubercle bacilli in milk. ×1000.

but not with the human type. In guinea-pigs and monkeys a generalised tuberculosis results from subcutaneous injection of bacilli of the human type, but in this case also the difference in favour of the greater virulence of the bovine type is made out. With regard to the distribution of the two types of organisms, it may be stated that, so far as we know, the bacillus obtained from bovine tuberculosis is practically always of the bovine type; in fact, this seems to be the prevalent organism in animal tuberculosis (*vide infra*). In human tuberculosis the bacilli in a large majority of the cases are of the human type; but, on the other hand, in a certain proportion bacilli of the bovine type are present. Pulmonary phthisis is, with few exceptions, caused by bacilli of the human type. Cases have been recorded in which the bovine type has been present, but these constitute less than 1 per cent. of the cases investigated. In Scotland, however, an incidence of 3·8 per cent. has been recorded. The Royal Commission found that the bovine type was present in 50 per cent. of cases of primary abdominal tuberculosis in children—that is, in cases where apparently infection had taken place by alimentation; more recent observations have shown that the proportion of cases of glandular tuberculosis in children under ten years of age produced by bovine bacilli varies considerably in different localities. In cases of lupus nearly half of the strains obtained are of the bovine type, and it is an interesting fact that many of them, both of the human and bovine types, have been found to be markedly attenuated in their virulence for animals. In over two hundred cases of tuberculosis in children, reported by W. H. Park, the bovine bacillus was present in more than

FIG. 78.—Cultures of bovine and human tubercle bacilli five weeks old on glycerol egg. The central tube is human the tubes on each side bovine. The three tubes were inoculated on the same day.

25 per cent., the percentage being higher in the earlier than in the later years of childhood ; and Fraser found that of seventy cases of tuberculosis of bones and joints in children in Edinburgh, this was the type present in more than half. Fraser also found that the proportion of cases in which the bovine type is present is much higher when there is no evidence of infection from other members of the family, than when there is the possibility of such infection. The majority of the tuberculous lesions from which the bovine type has been obtained have been in children. Blacklock in the West of Scotland investigated, *post mortem*, 434 consecutive cases of tuberculosis in children. The pathological evidence pointed to the respiratory system being the portal of entry in 283 (65·2 per cent.); the infecting organisms were isolated in 166, 160 being human strains and 6 (3·6 per cent.) bovine. Primary respiratory tuberculosis was very fatal in children under three years and slightly less so in children between three and thirteen. In 140 the primary site of infection was in the intestinal tract and from these 73 strains were isolated, 13 being human and 60 (82·2 per cent.) bovine. Primary abdominal tuberculosis was less often the cause of death than primary respiratory tuberculosis, but in abdominal infections also a higher mortality was noted in children under three years. In the remaining 16 cases of this series the portal of entry was in the cervical glands in 9 (2·07 per cent.) and in 7 cases the primary site of infection could not be discovered. More than half (37) of the bovine strains were isolated from children under two years of age. From surgical conditions in 54 children, strains were isolated as follows : tuberculous cervical adenitis, 10 human and 18 bovine (64·3 per cent.); tuberculous disease in bones and joints, 17 human and 9 bovine (34·6 per cent.): 52 of these strains were isolated during life, and 28 of them (53·8 per cent.) were of the bovine type.

The following collective figures given by A. S. Griffith indicate the percentage prevalence of the bovine type of tubercle bacillus in various forms of tuberculosis in this country : cervical gland tuberculosis, 85·7 in England and 84·9 in Scotland under five years of age; and 45·7 in England and 73·6 in Scotland at all ages: bone and joint tuberculosis, 18 in England and 42·8 in Scotland: meningitis, 26·7 in England and Scotland at all ages; lupus, 48·6 in England and 53·8 in Scotland : pulmonary tuberculosis, 0·8 in England and 3·8 in Scotland : genito-urinary tuberculosis, 17·4 in England. In Scotland, MacGregor, Kirkpatrick, and Craig have found the bovine type in 28 per cent., and Blacklock and Griffin in 22·5 per cent. of cases of tuber-

culous meningitis. Alston and Griffith have recorded a percentage prevalence of 30·9 bovine type tubercle bacilli in a series of cases of tuberculosis of the urinary system in Scotland.

In addition to these data regarding the relative prevalence of the bovine type of tubercle bacillus in the different forms of tuberculosis and at different ages, the following facts are of special significance: as estimated, at least 40 per cent. of dairy cows in this country are tuberculous, though only about 0·2 per cent. exhibit udder tuberculosis and actively discharge tubercle bacilli in their milk. As a result of mixing or bulking, an average of 6·7 per cent. of samples of raw market milk in the large communities contain living tubercle bacilli. All the evidence points to cows' milk as a serious source of human tuberculosis. It is estimated that in England and Wales about 2000 deaths annually are caused by the bovine type of bacillus and about 4000 new cases of this form of infection occur each year.

It has been held that in infancy the susceptibility to infection with the tubercle bacillus is so great that infection once established practically always runs an acutely fatal course; accordingly it was believed that latent tuberculosis did not occur at early ages. The accidental administration of a culture of living virulent tubercle bacilli by mouth (instead of B.C.G. vaccine) to 251 infants (in the first ten days of life) at Lübeck in 1930 has furnished evidence which makes these views untenable. Of these children 76 died within the first year, one in the second year, and none in the third year; in 72 of them (28·7 per cent. of the total), there was extensive tuberculosis. Two-thirds of the children survived and in the succeeding three to four years developed well, although many of them showed evidence on X-ray examination of extensive lesions with caseation and calcification of the mesenteric glands. As regards the effect of the site of the primary lesion and the subsequent course of the disease, it is noteworthy that almost all of those who developed extensive primary lung lesions died.

Although most of the bacilli which have been cultivated correspond to one of the two types, as above described, it is also to be noted that intermediate varieties are occasionally met with, though some of these on analysis have been found to be really due to a mixture of the two types. Griffith has recorded the occasional occurrence of the following aberrant forms: dysgonic human, attenuated dysgonic human, attenuated eugonic human, eugonic bovine, attenuated eugonic bovine and attenuated dysgonic bovine types. The attenuated forms lack the virulence for animals possessed by the typical strains. Such strains have not infre-

quently been isolated from the skin in cases of lupus. According to some observers, it is possible to modify bacilli of the human type by passing them through the bodies of certain animals, *e.g.* guinea-pigs, sheep, and goat, so that they acquire the characters of bovine bacilli, but the more recent results are that this modification does not take place and that the characters of the type are comparatively stable. Although the bovine bacilli are more virulent to the lower animals than the human bacilli are, this does not hold in the case of the human subject. In fact, the comparative chronicity of the primary abdominal lesions in children, in the first instance, would point rather to a lesser order of virulence towards the human subject. We may also add that there are cases, notably those of Ravenel, in which accidental inoculation of the skin in the human subject with bovine tubercle has resulted in the production of tuberculosis.

Some other facts may be given. The bovine type of bacillus is usually found in the sheep, goat, and horse, while in the pig the bovine type is found in the majority of cases, though occasionally the human type, and in a considerable proportion the avian tubercle bacillus, may be present. In the case of these two latter, the lesions are of a more localised kind. The avian type has also been isolated from the sheep and horse. The bovine type has been found in the cat. The human type is found in animals in confinement, *e g.* the chimpanzee and *Macacus rhesus*, and also in the parrot. The animals most susceptible to inoculation with the human type are the guinea-pig, *M. rhesus*, and chimpanzee; the dog, rat, and mouse are relatively resistant, while the calf, rabbit, pig, and goat occupy an intermediate position. The parrot also has been found to be susceptible to inoculation with the human type. It has been shown that when cows are inoculated subcutaneously with considerable quantities of bacilli either of the human or bovine type the bacilli are excreted in the milk, and that in these cases the udder appears normal. Tubercle bacilli have also been found in the milk of naturally infected cows in which there was no gross lesion of the udder (Gaiger and Davies). There is therefore the presumption that when during the course of the disease the bacilli are present in the blood stream, they may make the milk infective even though there are no discoverable lesions in the udder. The occurrence of infections of cows with the avian type has been recorded by Plum in Denmark; the uterus is specially affected and abortion results. Avian tubercle bacilli have also been isolated from cattle in Great Britain (Minett).

2. *Avian Tuberculosis.*—In the tuberculous lesions in birds there are found bacilli (*Mycobacterium avium*) which correspond in their staining reactions and in their morphological characters with those in mammals, but differences are observed in cultures, and also on experimental inoculation.

On glycerol agar and on serum, the growth of tubercle bacilli from birds is more luxuriant, has a moister appearance without the dry scaly character of the human type and, moreover, takes place at a higher temperature, 43° C., than is the case with mammalian tubercle bacilli. The optimum temperature is 41° to 42° C.

In glycerol broth a diffuse growth tends to occur in the depths of the medium. Experimental inoculation brings out even more distinct differences. Tubercle bacilli derived from the human subject or from the ox, for example, when injected into fowls, usually fail to produce tuberculosis, while those of avian origin very readily do so (on the other hand, the parrot is susceptible to inoculation with both mammalian types). Fowls are also very susceptible to the disease when fed with portions of the organs containing avian tubercle bacilli, but they can consume large quantities of phthisical sputum without becoming tuberculous (Straus, Wurtz, Nocard). Rabbits and mice are the only mammals susceptible to inoculation with avian tubercle bacilli, though others may succumb to toxic effects when large doses are used. In the case of the rabbit, intravenous injection results in the formation of greyish-white foci in the spleen, but no true tubercles are formed; subcutaneous inoculation leads to a peculiar chronic disease in joints, testes, etc., whilst the liver and spleen are free from lesions—a result not obtained with mammalian bacilli. *Guinea-pigs which are so highly susceptible to the mammalian tubercle bacilli are resistant to the avian type.* Only a local lesion is produced by experimental inoculation.

Interesting results have been obtained by Winn and Petroff in the dissociation of the avian tubercle bacillus: four variants designated "S," "FS" (flat smooth), "R" and "Ch" (chromogenic) were separated differing in their physical and chemical properties and as regards the tissue reactions produced by them. As in their studies of dissociation of the human and bovine types, they found that the S forms were the most virulent and the leucocytic response to them of the acute type; the R form and the chromogenic variant were comparatively avirulent, producing a chronic healing type of lesion.

There is, therefore, abundant evidence that the bacilli derived from the two classes of animals show important differences, and, reasoning from analogy, we might infer that probably the human subject also would be little susceptible to infection from avian tuberculosis; in fact, only rare cases have been recorded in the human subject in which the avian bacillus was apparently the causal agent. The question remains—Are these differences of a permanent character? Nocard found that mammalian bacilli of the human type, when kept within closed collodion sacs in the peritoneal cavities of fowls over a long period of time, acquired the characters of avian bacilli, but the Royal Commission, as the result of similar experiments, obtained no evidence of such transformation. It is accordingly not possible at present to give a definite answer to the question.

3. *Tuberculosis in the Fish.*—Bataillon, Dubard, and Terre cultivated from a tubercle-like disease in a carp, a bacillus (*Mycobacterium piscium*) which, in staining reaction and microscopic characters, closely resembles the tubercle bacillus. The lesion with which it was associated was an abundant growth of granula-

tion tissue in which numerous giant-cells were present. It forms, however, luxuriant growth at room temperature, the growth being thick and moist like that of avian tubercle bacilli (Fig. 80, *b*). Growth does not occur at the body temperature, though by gradual acclimatisation a small amount of growth has been obtained up to 36° C. Furthermore, the organism appears to undergo no multiplication when injected into the tissues of mammals, and attempts to modify this characteristic have so far been unsuccessful. Weber and Taute have cultivated a similar organism from mud, and also from organs of healthy frogs. It is thus probably to be regarded as a saprophyte which is only occasionally associated with disease in the fish.

According to the results of different experimenters, it is possible to modify human tubercle bacilli by allowing them to sojourn in the tissues of cold-blooded animals, *e.g.* the frog, blind-worm, etc., so that they flourish at lower temperatures. These results have, however, been called in question, as it has been stated the organisms obtained were not modified tubercle bacilli, but other acid-fast bacilli which may be found in the tissues of normal cold-blooded animals.

Non-Pathogenic Acid-fast Bacilli.—A number of bacilli presenting the same staining reaction as the tubercle bacilli have been discovered. Such bacilli have a comparatively wide distribution in nature, as they have been obtained from various species of grass, from butter and milk, from manure, and from the surfaces of animal bodies. Microscopically, they agree more or less closely with tubercle bacilli, though most of them are shorter and thicker; many of them show filamentous and branching forms under certain conditions of culture. Moreover, on injection, they may produce granulation tissue nodules which resemble tubercles, although on the whole there is a greater tendency to softening and suppuration, and usually the lesions are localised to the site of inoculation. The most important point of distinction is the fact that their multiplication on artificial media is much more rapid, growth usually being visible within forty-eight hours and often within twenty-four hours at 37° C. Furthermore, in most instances growth occurs at the room temperature and ordinary agar is a suitable medium. The general character of the cultures in this group is a somewhat irregular layer, often with wrinkled surface, dry or moist in appearance, and varying in tint from white to yellow or reddish-brown.

As examples of acid-fast saprophytes we may mention Moeller's grass bacillus (*Mycobacterium phlœi*) (isolated from infusions of Timothy grass), Petri's (*M. butyricum*), and Rabinowitsch's (*M. berolinense*) butter bacilli (Fig. 79), the "Mist" (dung) bacillus of Moeller (*M. stercoris*), etc. Kolle and his co-workers have stated that as a result of repeated animal passage certain of the sapro-

phytic acid-fast organisms became enhanced in virulence until they behaved like tubercle bacilli ; at the same time their cultural

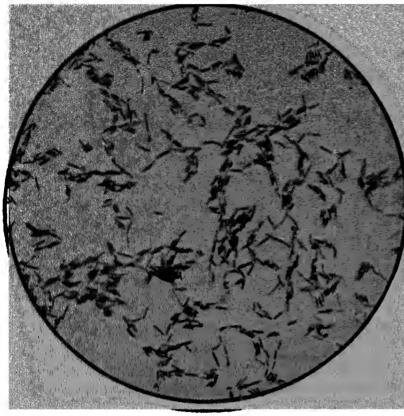


FIG. 79.—Moeller's Timothy-grass bacillus. From a culture on agar. Stained with carbol-fuchsin, and treated with 20 per cent. sulphuric acid ×1000.

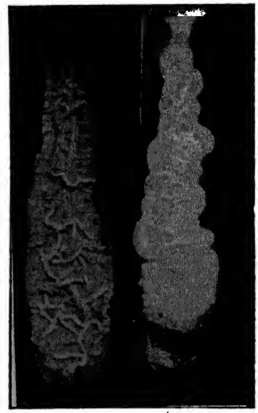


FIG. 80.—Cultures of acid-fast bacilli grown at room temperature.
(*a*) The Petri - Rabinowitsch butter bacillus.
(*b*) Bacillus of fish tuberculosis

characters also became altered so that finally they resembled the latter. It may be said, however, that the tubercle bacilli and the acid-fast saprophytic organisms are distinct species.

Smegma Bacillus (*Mycobacterium smegmatis*).—This organism is of importance, as in form and staining reaction it somewhat resembles the tubercle bacillus and may be mistaken for it. It occurs often in large numbers in smegma and in the region of the external genitals, especially where there is an accumulation of fatty matter from the secretions. Morphologically it is a slender, slightly curved organism, like the tubercle bacillus, but usually distinctly shorter (Fig. 81). It stains with some difficulty and resists decolorisation with strong mineral acids. Most observers

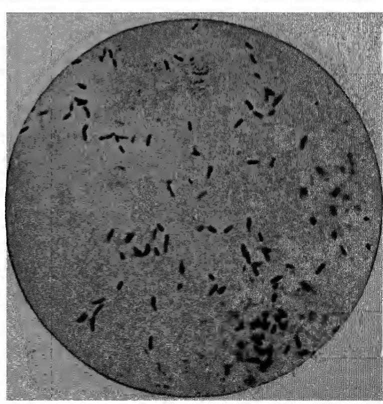


FIG. 81.—Smegma bacilli. Film preparation of smegma. Ziehl-Neelsen stain. ×1000.

ascribe the latter fact to the fatty matter with which it is surrounded, and find that if the specimen is treated with alcohol the organism is easily decolorised. Czaplewski, however, who has cultivated it on various media, finds that in culture it shows resistance to decolorisation both with alcohol and with acids, and considers, therefore, that the reaction is not due to the surrounding fatty medium. We have found that in smegma it can be readily decolorised, as a rule, by a minute's exposure to alcohol after the usual treatment with sulphuric acid, and thus it can be readily distinguished from the tubercle bacillus. We, moreover, believe that minor points of difference in the microscopic appearances of the two organisms are quite sufficient to make the experienced observer suspicious if he should meet with the smegma bacillus in urine, and lead him to apply the decolorising test. Difficulty will only occur when a few scattered bacilli retaining the fuchsin are found, or if they are seen to be enclosed in a matrix of hyaline material. The preparation of stained films after treatment of the urinary sediment with antiformin is of value in such cases, as organisms which lose their acid-fast properties in the process are not true tubercle bacilli.

Its cultivation was first effected by Czaplewski. On serum it grew in the form of yellowish-grey, irregularly rounded colonies about 1 mm. in diameter, sometimes becoming confluent to form a comparatively thick layer. He found that it also grew on glycerol agar and in broth. Its cultural characters generally resemble those of the saprophytic acid-fast bacilli described above. It is non-pathogenic to various animals which have been tested, unless very large doses are used.

Cowie has found that acid-fast bacilli are of common occurrence in the secretions of the external genitals, mammæ, etc., in certain of the lower animals, and that these organisms vary in appearance He considers that the term "smegma bacillus" probably represents a number of allied species.

The occurrence of saprophytic and commensal acid-fast bacilli must be borne in mind in utilising the reaction of acid-fastness for the identification of the tubercle bacillus. The source of any acid-fast bacilli in question is manifestly of importance, and it may be stated that when these have been obtained from some source outside the body, or where contamination from without has been possible, their recognition as tubercle bacilli cannot be established by microscopic examination alone. In the case of material coming from the interior of the body, however—sputum, etc.—the condition must be looked on as different, and although an acid-fast bacillus (not the tubercle bacillus) has been found by Rabinowitsch in a case of pulmonary gangrene, we have not sufficient data for saying that acid-fast bacilli other than the tubercle bacillus (and the leprosy bacillus) flourish within the tissues of the *human* body, except in such rare instances as to be practically negligible. Accordingly, up till now, the microscopic examination of sputum, etc., cannot be said to have its validity shaken, and the results of clinical

experience show that such examination is of practically unvarying value. Nevertheless, the facts established with regard to other acid-fast bacilli must be kept carefully in view, and great care must be exercised when only one or two bacilli are found, especially if they deviate in their morphological characters from the tubercle bacillus. In such cases inoculation may be the only reliable test.

Practical Conclusions.—From the facts above stated with regard to the conditions of growth of the tubercle bacilli, their powers of resistance, and the paths by which they can enter the body and produce disease (as shown by experiment), the manner by which tuberculosis is naturally transmitted can be readily understood. The tubercle bacilli leave the body in large numbers in the sputum of phthisical patients. As examples of the extent to which this takes place, it may be said that their presence in the air of rooms containing phthisical patients has been repeatedly demonstrated. Williams placed glass plates covered with glycerol in the ventilating shaft of the Brompton Hospital, and after five days found, by microscopic examination, tubercle bacilli on the surface, whilst Klein found that guinea-pigs kept in the ventilating shaft became tuberculous. Cornet produced tuberculosis in rabbits by inoculating them with dust collected from the walls of a consumptive ward. So far as the human subject is concerned therefore, the great means of disseminating the bacilli in the outer world is dried phthisical sputum, and the source of danger from this means can scarcely be overestimated. The bacilli, of course, are present in the minute droplets expelled from the mouth of the patient when coughing. Tubercle bacilli are also discharged in considerable quantities in the urine in tuberculous disease of the urinary tract, and by the bowel when there is tuberculous ulceration.

Another great source of infection is the milk of tuberculous cows, especially those affected with tuberculosis of the udder, and this is responsible for a considerable proportion of tuberculosis of lymphatic glands, bones, and joints, etc., in young children, as above detailed. In the examination of milk, animal inoculation with centrifuged samples is the most reliable means of detecting the presence of tubercle bacilli. The milk from cows thus affected is probably the great source of tabes mesenterica, which is so common in young subjects (*vide* p. 423). In these cases there may be tuberculous ulceration of the intestine, or it may be absent. It is especially in children that this mode of infection occurs, as in the adult ulceration of the intestine is rare as a primary infection, though it may result in

phthisical patients from infection by the bacilli in the sputum which has been swallowed. There is less risk of infection by means of the flesh of tuberculous animals, for, in the first place, tuberculosis of the muscles of oxen being very rare, there is little chance of the bacilli being present in the flesh unless an affected lymph gland is included or the surface has been smeared with material from tuberculous organs, as in the process of cutting up the parts; and, in the second place, even when present they will be destroyed if the meat is thoroughly cooked.

We may state, therefore, that the two great modes of infection are by inhalation, and by ingestion, of tubercle bacilli. In the former, the tubercle bacilli will in most cases be derived from the human subject; in the latter, probably from tuberculous cows, though inhaled tubercle bacilli may also be swallowed and contamination of food by tuberculous material from the human subject may occur. Alike when inhaled and when ingested, tubercle bacilli may lodge about the pharynx and thus come to infect the pharyngeal lymphoid tissue, tonsils, etc., tuberculous lesions of these parts being much more frequent than was formerly supposed. Thence the cervical lymphatic glands may become infected, and afterwards other groups of glands, bones, or joints, and internal organs. It is to be noted that there is a predilection for the lungs by whatever route the bacilli enter the body, and accordingly that pulmonary lesions are not always the result of inhalation.

Specific Reactions in Tuberculosis; Supersensitiveness, or Allergy.—The account of the effects of the tubercle bacillus given above shows that it produces toxic substances which both act on the tissues around and also affect the body generally. Nevertheless the organism does not to any extent produce separable soluble toxins in fluid media; the toxic substances are bound up with the substance of the bacteria, that is, are endotoxins. Tuberculin as first introduced by Koch in 1890 consisted of the disintegration products of the bacilli and thus contained endotoxins along with other substances. He found that in tuberculous animals there was supersensitiveness to tuberculin, which was shown by a *local* reaction at the site of introduction of the tuberculin, by a *general* reaction, indicated by malaise, pyrexia, etc., and also by a *focal* inflammatory reaction around the tuberculous lesions. These reactions accordingly supply a means by which the presence of tuberculous foci in the body can be recognised. When a sufficiently large dose of tuberculin (0·2—0·5 c.c.) is injected subcutaneously into a guinea-pig which has become infected following inoculation

with tubercle bacilli four or five weeks previously, the animal dies in six to thirty hours and exhibits *post mortem* a severe inflammatory reaction with hæmorrhage at the sites of tuberculous lesions. The condition differs from acute anaphylactic shock especially in respect of the long interval before death; also passive transference of supersensitiveness to tuberculin does not appear to have been effected.

Koch supposed that the focal reaction might lead to encapsulation of the tuberculous lesion and thus to cure, or, if the lesion were superficial, to its being ulcerated and cast off. He advocated tuberculin for therapeutic as well as diagnostic purposes. It soon came to be recognised, however, that the administration of tuberculin in the doses then used (doses sufficient to produce a general reaction) might be attended by harm, and the use of tuberculin in this way was abandoned. At a later period treatment by tuberculin was resumed but was carried out with very much smaller doses, in fact with doses insufficient to produce systemic disturbance; and favourable results have been claimed, though the therapeutic value of tuberculin has been questioned by many clinical observers. Tuberculin employed in this way might possibly act as a stimulant of the tissue around the tuberculous lesions and thus be beneficial; it might aid also by leading to the production of specific antibodies. But in any case it must be regarded as only an auxiliary agent, and even then only when employed with care and over a considerable period. (For varieties of tuberculin, *vide* p. 441.)

Tuberculin Reactions.—The original method employed by Koch for eliciting the tuberculin reaction was that of subcutaneous injection, and with it all types of reaction—local, general, and focal—are seen.

Subcutaneous method (Koch).—A positive reaction may be produced in tuberculous subjects by the injection of 0·001–0·0001 c.c. of the "old" tuberculin. A local inflammatory swelling occurs at the site of injection, and this is accompanied by a rise of temperature, usually of a short character. There is also a focal reaction around any tuberculous lesion, and if the lesion is visible, *e.g.* lupus, this is indicated by an inflammatory redness, which may be followed by ulceration. In the case of a pulmonary lesion there may be signs of irritation—tendency to cough, increased expectoration, increase of râles in the chest, etc. This method is not used now for diagnostic purposes in the human subject.

Cutaneous Test of Pirquet.—This is carried out as follows: The skin, usually that of the flexor aspect of the forearm, is well cleansed with ether and then allowed to dry. Two drops of

tuberculin are placed on the prepared surface about 4 inches apart, and then midway between the two drops a small spot is scarified with a small metal borer constructed for the purpose ; in the process only the epidermis should be injured and blood should not be drawn. This serves as a control, any reaction which follows in it being merely a traumatic one. Similar scarification is effected through the drops of tuberculin, so that the scarified spots are exposed to its action. Small portions of cotton wool are placed over the drops to prevent the tuberculin from running off, and the latter is allowed to act for ten minutes. After that time the cotton wool is removed ; no dressing is required. The " old " tuberculin of Koch is that used. In the case of a positive reaction an inflammatory redness and swelling make their appearance round the sites of tuberculin inoculation, generally within a few hours, and at the end of twenty-four hours there is a distinct inflammatory papule about half an inch in diameter, with a somewhat paler centre like a spot of urticaria ; sometimes in the centre there are minute vesicles. The maximum effect usually occurs within forty-eight hours, and after that time the reaction gradually recedes. Such is the typical reaction, but of course slighter, and also more intense reactions are met with. In a negative reaction all three points of scarification show merely a slight traumatic redness which soon passes off.

Intracutaneous Method of Mantoux.—In this modification 0·1 c.c. of diluted tuberculin is injected into the cutis by means of a fine needle. The method of successive graded injections is specially recommended. For the initial dose a 1 : 10,000 dilution of tuberculin in 0·5 per cent. phenol-saline is used. Should this fail to cause a positive reaction consisting of an area of erythema or erythematous infiltration not less than 5 mm. in its greatest diameter in forty-eight to ninety-six hours, the test is repeated with a 1 : 1000 dilution. If the result be again negative a re-test is carried out with 1 : 100 tuberculin and, if negative again, with a 1 : 10 dilution. Re-tests should be made either within a week or after an interval of several months following the previous injection in order to avoid the possibility of sensitisation causing a violent reaction in the intervening period.

The Ophthalmo-reaction of Calmette.—A dilute solution of " old " tuberculin, after purification by precipitation with alcohol, is instilled into the eye. A positive result consists in an acute inflammatory reaction of the conjunctiva, which reaches its maximum usually six to ten hours later.

The general results obtained by these reactions appear to correspond closely. A distinct positive result obtained by either is nearly conclusive as to the presence of a tuberculous lesion, though not necessarily of active nature. In cases of latent tuberculosis the reaction is sometimes obtained, sometimes not. Again, in very advanced cases of tuberculosis, especially a short time before death, a negative result may be got; in some of these cases Pirquet met with a colourless papule or a livid spot without exudation, conditions which he described as indicating a "cachectic reaction." Also, in measles the skin is temporarily insensitive to tuberculin. The cutaneous and intracutaneous methods are those which are now generally used. The former is the more readily applied, but the latter is the more delicate. The ophthalmo-reaction also is easily applied, at least in adults, but its use is contra-indicated when there is any abnormal condition of the conjunctiva. Even apart from this, however, inflammatory symptoms of disagreeable severity sometimes supervene, and the test is now not much used. The tuberculin reaction is an example of allergy (p. 268). The behaviour of the general population in Europe and America has now been extensively investigated by means of the tuberculin reaction. It has been found that while the new-born are insensitive even to large doses, as age advances the number of those who react positively increases steadily until in adult life it becomes very high, reaching 80 per cent. or more, according to the particular country and to whether an urban or a rural district is examined. These results taken along with the pathological evidence, which tends in the same direction, indicate that a great proportion of the population ultimately becomes infected and that, accordingly, the prevalence of tuberculous infection is much higher than purely clinical observations would suggest. The value of the tuberculin reaction as an indication of active infection is therefore greatest in very young children, since the evidence points to latent tuberculosis being uncommon in them. It is generally agreed that while infections with human or bovine strains react practically equally to tuberculins prepared from either type of tubercle bacilli, they react much less intensely to avian tuberculin and *vice versa*.

The Use of Old Tuberculin in the Diagnosis of Tuberculosis in Cattle.—In cattle, tuberculosis may be present without giving rise to apparent symptoms. It is thus important from the point of view of human infection that an early diagnosis should be made. The method is applied as follows: The animals are kept twenty-four hours in their stalls, and the temperature is taken every three hours,

from four hours before the injection till twenty-four after. The average temperature in cattle is 102·2° F.; 30 to 40 centigrams of tuberculin are injected subcutaneously, and if the animal be tuberculous the temperature rises 2° or 3° F. in eight to twelve hours, and continues elevated for ten to twelve hours. Bang, who has worked most at the subject, laid down the principle that the more nearly the temperature approaches 104° F. the more reason for suspicion is there. He gave a record of 280 cases where the value of the method was tested by subsequent post-mortem examination. He found that with proper precautions the error was only 3·3 per cent. The method has been largely practised in all parts of the world, and is of great value. It may be noted that if a positive reaction is present it may disappear after repeated injections.

The *double intradermal test* introduced by the Tuberculin Committee of the Medical Research Council has been widely accepted as yielding results more trustworthy and easy to interpret than the subcutaneous method. In this procedure a fold of shaved skin near the middle of the animal's neck is taken up between the thumb and forefinger of the left hand, and its thickness is measured with calipers; then 0·1 c.c. undiluted old tuberculin is injected as deeply as possible without penetrating into the subcutaneous tissue. At the end of forty-eight hours the character and thickness of any swelling at the site of injection are noted. A positive result is indicated by a large diffuse swelling with ill-defined edges, which is hot and tender. If, however, there is only a hard infiltration of the size of a pea or bean, without heat or tenderness, the result is inconclusive, and a second injection of the same dose is required; the latter is made at once into the centre of the infiltrated area. The result is read after twenty-four hours; a positive reaction is shown by an inflammatory swelling with the same characters as those observed in animals which react positively to the first injection. By this method, out of 835 animals which had shown no reaction to the subcutaneous test, 122 reacted positively to the double intradermal test; 94 of the latter came to examination *post mortem*, and all of these were found to be tuberculous.

Immunity Phenomena.—In tuberculosis we meet with certain phenomena unlike those in acute infections against which a solid immunity may be acquired. In the first place, the local reaction around the bacilli plays a very important part, as it represents a struggle between the tissues and the bacilli, in which either may be successful. It is now a well-recognised fact that infection and subsequent cure take place in the early years of life in a large proportion of individuals, and that cure may follow even when the lesion has been of considerable extent. The tissue reaction around the bacilli tends to localise them and also their products, and, at the same time, the endotoxins are bound up in the resistant bodies of the bacteria. The conditicus for diffusion of bacterial products are thus unfavourable, and it may be that healing results merely from local reaction without a general immunity being established. Nevertheless,

the facts with regard to the tuberculin reaction point to a general effect on the tissues of the body brought about by the bacillary products, and it is generally assumed that healing of tuberculosis in early life brings with it a certain degree of immunity. Evidence of healed tuberculosis, usually in the lungs, is found in adults, and it is supposed that the appearance of active disease may be due to the lighting up of a dormant lesion. It is also frequently due, however, to fresh infection, and this is indicated by the fact that in practically all cases of adult tuberculosis the bacilli present are of the human type. In either case, in the development of active disease depressed vitality from under-nutrition and like conditions are recognised as important factors, overcoming any immunity and leading to susceptibility. It is possible that the result may vary also according to the virulence of the bacilli, but with regard to this less is known.

The serum of individuals affected with tuberculosis has been investigated for the presence of specific antibodies; and agglutinins, precipitins, opsonins, and complement-fixing immune-bodies have all been demonstrated. Bordet and Gengou showed originally that guinea-pigs which had received subcutaneous injections of avian tubercle bacilli (to which these animals are relatively insusceptible), developed in their serum complement-fixing antibodies for both avian and human types of bacilli. A similar result followed injections of the human type killed by heating at 70° C. and later of dried bacilli. On the other hand, no antibodies were demonstrable at any stage of the infection in animals inoculated with living, virulent, human bacilli. It has been found that complement-fixing antibodies may occur in the serum of human cases of tuberculosis, and that they occur in largest amount in chronic infections of fairly wide extent. On the other hand, they may be absent when the lesion is encapsulated or quiescent, and also when the disease is advancing rapidly, and especially towards the fatal termination. The results obtained, however, have not been such as to be of much clinical value.

While agglutinins may be demonstrated in the blood of tuberculous patients, it is not possible to correlate the agglutinating power with the degree of immunity present. Agglutinins obtained by injecting animals with dead bacilli have been employed for another purpose, namely, to compare different strains of bacilli. Tulloch and his co-workers found that the strains of the human tubercle bacillus formed one well-defined serological group, there being no evidence of the existence of multi-

plicity of types. G. S. Wilson came to the same conclusion, and found also that bovine and human strains are indistinguishable by agglutination tests. It has also been thought that the essential mechanism of immunity may be opsonic in nature and at one time tuberculin was used for therapeutic purposes with the object of increasing the opsonic power of the serum towards the tubercle bacillus, the treatment being controlled by the opsonic index (*vide* p. 143). It may be added that antibodies may result from the injection of large doses of tuberculin in normal animals. But it is not clear whether, and if so to what extent, the presence of antibodies can be taken as an indication of increased resistance to tuberculosis. In this connection it is of interest to note that Laidlaw and Dudley found that the gum-like substance extracted by them from tubercle bacilli gave a precipitate with a tubercle antiserum. This substance did not, however, act as an antigen, no precipitin being developed when it was injected into animals. In this respect it acted like a hapten (*cf.* the specific polysaccharides of the pneumococcus).

It may be taken as established that to confer any considerable degree of acquired immunity against tuberculosis, it is necessary to inoculate the individual with living, although attenuated, tubercle bacilli; and this resistance probably disappears soon after the body ceases to harbour the living organisms. In accordance with this view, many attempts have been made to attenuate tubercle bacilli in various ways. Calmette and his co-workers have obtained successful results by immunising animals with a culture of bovine tubercle bacilli which have been grown for many years on a medium containing bile; this is generally known as culture "BCG" (*Bacille Calmette-Guérin*). In the new-born, according to Calmette, the alimentary mucosa is highly permeable, and the vaccine may be administered by mouth as well as intravenously. Immunity conferred by the use of such attenuated cultures is of relatively short duration, about eighteen months, in cattle. Wilbert's observations indicated that Calmette's method affords protection to monkeys in confinement against natural infection by contact from tuberculous animals. Griffith, however, has found that the immunity produced is at most of a low grade. These results go to support the view that the healing of a tuberculous lesion in earlier life leads to a certain amount of immunity. They are also in accordance with the fact first brought forward by Koch that a tuberculous animal reacts differently from a normal animal to a fresh infection. In the former the resulting lesions remain ocalised, while in the latter there may be a spreading infection.

Römer showed that tuberculous guinea-pigs were resistant to a small reinfection; and he emphasised that the state of supersensitiveness due to the primary infection brought about a rapid and violent reaction to a superimposed infection with healing of the new focus. He assumed that in man mild superinfections are frequently aborted in the same way, though a massive re-infection may overcome the resistance and lead to ulcerative tuberculosis of the lung. Many other observers have also been inclined to correlate resistance with allergy. In this connection it may be noted that dead bacilli produce only a transitory supersensitiveness as compared with the typical allergic condition resulting from infection by living organisms, and as pointed out above, dead organisms have little or no immunising action. It has been supposed that the allergic response to a new infection brings about a rapid mobilisation of the same defensive forces which operate in the normal animal (see Long). The evidence also indicates that phagocytosis is more active in the allergic individual. On the other hand, certain observers have claimed that allergy and immunity are not necessarily correlated and that the supersensitive state may, in reality, be an adverse factor (Rich). Thus Sewall and his co-workers have found that in guinea-pigs superinfected after varying times following a primary infection resistance tends to be inversely proportional to cutaneous allergy. Although at present no definite conclusions can be formulated regarding this question and though allergy may not necessarily indicate increased resistance, nevertheless it is clear that allergy represents one phase of the reactive mechanism on which immunity depends.

Active Immunisation of the Human Subject.—In view of the apparent innocuousness of his culture BCG (*vide supra*) for the various species of animals susceptible to tuberculosis, Calmette initiated the immunisation of infants with it. Several doses were given by mouth within the first ten days after birth, the object being to confer protection at the most susceptible period. On experimental grounds, immunity is believed to be well developed a month later. Objection has been taken to the procedure on the grounds both of its efficacy and its safety. As regards the efficacy of the treatment, Calmette's favourable statistics have been criticised by Greenwood and others, and it has been concluded that the value of the method is still unproved. On the other hand, striking observations have been recorded by Heimbeck as to its protective value in the case of nurses, originally reacting negatively to tuberculin, who were injected with the vaccine before taking up duty in hospital

so as to render them positively reacting to the Pirquet test. Park and his co-workers have analysed the records of 307 children vaccinated with BCG along with 453 controls and have been unable to draw any definite conclusions as to the degree or duration of its protective action. The analogy, however, of the immunity to tuberculous infection conferred by BCG on calves and other animals cannot be disregarded. Immunisation of calves by BCG has been the subject of a series of observations in recent years by Griffith and Buxton. In their earlier experiments in which the animals were vaccinated by different routes, alimentary, tracheal, subcutaneous and intravenous, and were subsequently inoculated intravenously with fully virulent bovine tubercle bacilli, increased resistance was clearly demonstrated after intravenous vaccination, while vaccination by the other routes was of doubtful or variable efficacy. In all the experiments there was great individual variation in the degree of resistance. In subsequent tests in which a double intravenous injection of the vaccine was given, it was found that three months after vaccination the resistance against orally administered virulent organisms was complete. In later experiments the duration of the increased resistance was estimated: as before, a complete immunity to oral administration of virulent organisms was demonstrated three months after vaccination; at six months the protection was almost complete, but after nine and twelve months a progressive decrease of resistance was noted. Moreover, further vaccination did not completely restore the declining immunity. It has thus been possible to produce an effective resistance, though of somewhat limited duration, by intravenous vaccination with BCG.

As regards safety, the use of a living attenuated culture involves the risk that virulence may be regained. In calves and oxen the culture appears to be devoid of pathogenic action (Gerlach), but in small animals such as guinea-pigs tuberculosis may be produced by its injection. Petroff and his co-workers isolated different types of colony from the culture and have produced fatal tuberculosis in guinea-pigs; a similar result was obtained by Begbie, who found also that the smooth form was the most virulent. Dreyer and Vollum claimed that by serial culture of BCG in the depth of a veal broth of pH 6·8 they restored its virulence for the guinea-pig and rabbit. Other workers have failed to separate virulent strains by dissociation from BCG cultures. Lurie has found in experiments with rabbits that the organisms multiply in the tissues but are soon destroyed; some, however, persist in the lymph nodes without

producing changes in the tissue. He states that though typical lesions may be produced, these resolve completely. Calmette collected the records of inoculation of a million children without the production of ill effects. It seems, however, that the possibility of a return of virulence under conditions as yet undefined cannot be excluded. A minor accident attending subcutaneous injections is the development of cold abscesses at the sites.

Spahlinger Vaccine.—A vaccine of the tubercle bacillus prepared by growing it directly from the body on medium made from the body fluids of the animal species in which the organism has been flourishing under natural conditions has been advocated by Spahlinger. The supposed object is to obtain *in vitro* antigens identical to those generated by the organism in the living body of the infected individual. In addition, no chemical or physical reaction or manipulation is employed which might, as Spahlinger maintains, denature the specific antigens. Thus the organisms after being emulsified from the culture are kept until they have died a " natural " death in the course of time, *e.g.* for a year or longer. Recently experiments on the protective value of Spahlinger's vaccine in calves have been carried out by the Government of Northern Ireland. The results can hardly be regarded as conclusive, though they suggest that immunity is produced by the vaccine. Further investigation is required with regard to the supposed principle on which the preparation of the vaccine is based, the actual efficacy of the vaccine and the viability of the organisms contained in it.

Varieties of Tuberculin.—(1) *Koch's Old Tuberculin.*—This consists of a six-weeks-old culture of tubercle bacilli in 5 per cent glycerol broth, evaporated down to a tenth of its original volume killed by heat, and filtered.

Standardisation of Tuberculin.[1]—The potency of preparations of tuberculin is determined by comparing their effect on tuberculous guinea-pigs with that of a standard product. This may be done by injecting intracutaneously 0·2 c.c. of varying dilutions of the standard specimen and of that under test. In order to obtain reliable results, a series of at least six guinea-pigs is used ; readings are made eighteen to twenty-four hours after the injection. The standard tuberculin should produce an obvious reaction in a dilution of 1 : 2000 to 1 : 4000. Another method consists in selecting tuberculous guinea-pigs at a stage of the infection when a subcutaneous injection of 0·1 c.c. standard tuberculin kills 50 per cent. of the animals. That concentration of the preparation under test of which 0·1 c.c. subcutaneously produces a similar mortality is taken as equivalent to the standard. The subcutaneous injection of 0·5 c.c. of old tuberculin causes no ill effects in a normal guinea-pig.

(2) *Tuberculin-O.*—Masses of living bacillary growth from surface cultures on agar are dried *in vacuo*, ground in an agate mill,

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[1] The *Therapeutic Substances Regulations*, prescribe that tuberculin before being supplied commercially must be standardised by an approved method.

treated with distilled water and centrifuged; the supernatant clear fluid is the tuberculin. As it gave no cloudiness on the addition of glycerol, Koch concluded that it contained the glycerol-soluble products present in the "old tuberculin," which were looked on as responsible for the necrotic effects produced by the latter (*vide supra*).

(3) *Tuberculin-R.*—The deposit in the preparation of tuberculin-O is again ground up in distilled water, centrifuged, and the clear fluid set aside; the process is repeated with the residue until, on centrifuging, none is left. The successive supernatant fluids are mixed and concentrated, and constitute the tuberculin. As this fluid gives a cloudiness with glycerol, Koch considered it contained the glycerol-insoluble constituents of the "old tuberculin."

(4) *Koch's New Tuberculin* (*Bazillenemulsion*).—A bacillary mass is dried and ground in 50 per cent. glycerol in water till a clear fluid results. This tuberculin is thus equivalent to a mixture of tuberculin-O and tuberculin-R.

(5) *Tuberculin from Cultures on Synthetic Media.*—A number of other tuberculin preparations have been used, but the above are the most important. By using cultures of tubercle bacilli on synthetic media a tuberculin preparation can be obtained which is free from any non-specific action such as may be produced by proteins derived from the usual culture media (Douglas and Hartley). It has also been shown that the specific protein of the tubercle bacillus on which the tuberculin reaction depends can be prepared in purified form and used as a tuberculin for clinical tests (Clark *et al.*: Seibert and Munday). These facts support the specific nature of the active substances in tuberculin. Long and Seibert have devised the following nutrient solution of known concentration which yields an abundant growth of the tubercle bacillus:

Asparagine	5	grams.
Ammonium citrate	5	,,
Potassium acid phosphate . .	3	,,
Sodium carbonate (anhydrous) . .	3	,,
Sodium chloride	2	,,
Magnesium sulphate . . .	1	,,
Ferric ammonium citrate . .	0·05	,,
Glycerol	50	,,
Water	1000	,,

Anti-Tuberculous Sera.—From what has been said regarding immunity reactions in tuberculosis it will be gathered that it is questionable whether the use of passive immunity in the treatment of tuberculosis has a rational basis. Several investigators, however, have introduced the sera of animals treated with the products of tubercle bacilli for therapeutic purposes. The clinical efficacy of these antisera has not been established.

Methods of Examination.—(1) *Microscopic Examination.*—Tuberculosis is one of the comparatively few diseases in which a diagnosis can usually be definitely made by microscopic examination alone. In the case of sputum, one of the yellowish fragments which are often present ought to be selected; dried films are then prepared in the usual way and stained by the Ziehl-Neelsen stain (p. 120). When tubercle bacilli are not found after ten to fifteen minutes' examination of a stained film, instead of prolonging the

search, it is advisable to examine further specimens (see Pottenger) (Young children habitually swallow the sputum; in their case stomach washings or fæces may be examined, or the examiner's finger, protected by a rubber cap, may be passed to the back of the pharynx and a specimen of sputum so obtained.) In the case of urine or other fluids, a deposit should first be obtained by centrifuging. Film preparations are then made from the deposit and treated as before. To avoid risk of contamination with the smegma bacillus, the meatus of the urethra should be cleansed and the urine first passed should be rejected, or the urine may be drawn off with a sterile catheter · As stated above, it is only exceptionally that difficulty will arise to the experienced observer. The best results in the examination of urine are obtained by using the sediment of a twenty-four hours' sample and treating it with antiformin (*vide infra*). Cerebro-spinal fluid is centrifuged and films are made from the sediment, but if a coagulum forms in the specimen, films should be made from it and may give better results than the centrifuged deposit. (For points to be attended to, *vide* p. 430.) The detection of tubercle bacilli by microscopical methods in sputum, pus, fæces, and even tissues, has been greatly facilitated by the introduction of the preparation called "antiformin." This is a mixture of equal parts of a solution of chlorinated soda (B.P.) and of a 15 per cent solution of caustic soda. It has a remarkable disintegrative and dissolving action on the tissues, etc., so that after it has been allowed to act on sputum, for example, and the mixture is centrifuged, the resulting deposit is scanty and the tubercle bacilli, if present, are accordingly greatly concentrated. The time necessary may be judged of by the appearance of the mixture, but it will generally be found that the desired result will be obtained after about an hour if 1 part of sputum be added to 2 or 3 parts of 20 per cent. antiformin; the mixture should be shaken from time to time, especially when the sputum is tenacious.

It should be noted that saprophytic acid-fast bacilli may occur in distilled water as used in the laboratory and in tap-water and often in smears from laboratory water-taps. This introduces the possibility of fallacy where water is used for microscopic preparations, *e.g.* from cultures. Containers for sputum, etc. should never be closed with corks, as these tend to harbour acid-fast organisms. Further, unused well-cleaned slides should be employed, since tubercle bacilli deposited on the glass are difficult to remove.

(2) *Inoculation.*—The guinea-pig is the most suitable animal. If the material to be tested is a fluid, it is injected subcutaneously or into the peritoneum; if solid or semi-solid, it is placed in a small pocket in the skin, or it may be thoroughly broken up in sterile water or other fluid and the emulsion injected. Intramuscular inoculation in the thigh is advantageous, as there is then little tendency for the local lesion to ulcerate. By these methods material in which no tubercle bacilli can be found microscopically may sometimes be shown to be tuberculous. Where other organisms are present, preliminary treatment with antiformin is advisable. A negative result of the inoculation test should not be recorded unless the animal has survived six weeks and no lesions are present.

The following method of G. Haswell Wilson for treating tissues is to be recommended: The tissue to be investigated is cut up into

small pieces with scissors, and is thoroughly rubbed up in a mortar with a small amount of dry sterile quartz sand. The rubbing up is continued till the fibrous tissue is disintegrated as far as possible, and the material forms a slightly moist, crumbling mass. The contents of the mortar are then washed into a wide test-tube with 15 to 20 c.c. of sterile saline solution. The sand is allowed to sediment for a few minutes ; as it falls, it carries down with it any coarser particles of tissue which remain. The supernatant fine suspension is then pipetted off, and thoroughly mixed with an equal volume of 15 per cent. antiformin. After five minutes, during which it should be stirred continuously, the mixture is centrifuged at high speed for a few minutes, and the supernatant fluid is discarded. The sediment is shaken up with sterile saline solution and again centrifuged. The shaking up of the sediment with sterile saline solution and centrifuging are carried out three times in all, so that no trace of antiformin remains. The sediment resulting from the final centrifuging is used for making cultures (see below), or, after emulsifying with a convenient amount of sterile saline solution, is injected into a suitable animal.

Lesions in inoculated animals must always be examined for the presence of the characteristic acid-fast bacilli, since pseudo-tuberculosis produces changes, usually in the liver and spleen, which to the naked eye resemble those of tuberculosis.

(3) *Cultivation.*—The surest method of obtaining pure cultures is to produce tuberculosis in a guinea-pig by inoculation with tuberculous material, and then, killing the animal after four or five weeks, to inoculate tubes of egg medium, under strict aseptic precautions, with portions of a tuberculous organ, *e.g.* the spleen. The portions of tissue should be fairly large, and should be well rubbed into the broken surface of the medium. Cultures may, however, be obtained from sputum, tissues, etc. after treatment by antiformin as described in paragraph (2). If, then, inoculations be made from the deposit on egg medium and glycerol egg medium, pure cultures of the tubercle bacillus may be obtained. The method is one which gives good results. The Löwenstein-Jensen medium may be specially recommended for obtaining primary growths of the human type of tubercle bacillus.

Petroff's method (modified) is also recommended as giving satisfactory results. In this, sputum is shaken with three to four times its volume of 4 per cent. caustic soda solution, and the mixture is placed for half an hour in the incubator at 37° C., shaking being repeated. At the end of this time it is centrifuged for half an hour at 3000 r.p.m. The supernatant fluid is then poured off and the sediment carefully neutralised to litmus paper with 8 per cent. HCl added drop by drop. The deposit is finally inoculated on egg medium containing 1 : 10,000 crystal violet or Löwenstein-Jensen medium.

Corper's Method.—One c.c. of sputum or other material, in the form of a homogeneous pulp, is mixed in a sterile 15 c.c centrifuge tube with 1 c.c. of a dilution of sulphuric acid made by adding slowly 17 c.c. concentrated acid, sp.g. 1·84, to distilled water till the final volume is 500 c.c. Mix thoroughly, stopper the tube, and keep at 37° C. for thirty minutes, shaking several times ; then dilute with 10 c.c. sterile 0·85 per cent. NaCl solution, mix well, and centrifuge. Make cultures from the sediment. Alternatively,

after treatment with acid, the centrifugalised deposit is neutralised with caustic soda and cultures then made.

(4) *Reactive Phenomena.*—The tuberculin reaction, along with the methods of applying the test, have been described above (p. **433** *et seq.*).

Some use has been made of the complement-fixation reaction with patient's serum for diagnostic purposes, though this test has only a limited practical application. The principle of the reaction is similar to that of other complement-fixation phenomena and the system adopted in the Wassermann test may be followed (*vide* p. 153 *et seq.*). The antigen is prepared from cultures of the tubercle

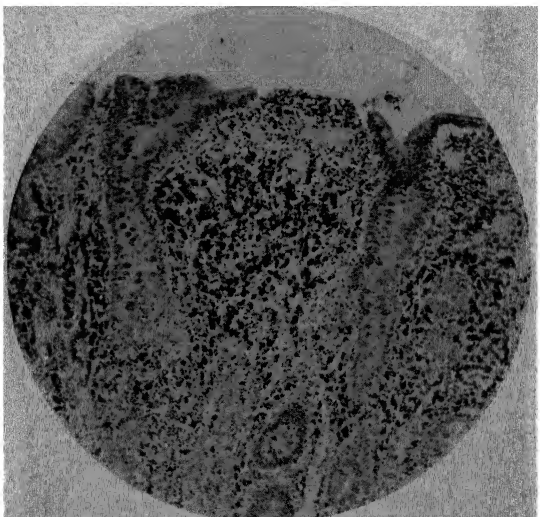


FIG. 82.—Section of intestine of ox in Johne's disease, showing the cells packed with bacilli. Ziehl-Neelsen stain. ×1000.

bacillus ; a preparation which has been frequently used is that of Besredka, obtained from cultures in egg broth. A suspension of tubercle bacilli from a culture may also be used (see Coulthard). It should be noted that tubercle antigens may give complement-fixation reactions with Wassermann-positive sera and this possibility must be allowed for by carrying out a parallel Wassermann test.

Identification of Type.—This depends chiefly on the pathogenic action of pure cultures, which are obtained either (1) directly from pathological products, commonly after treatment with antiformin, or (2) from an animal (guinea-pig) which has been inoculated with such materials. An emulsion of a young culture (two to three weeks) is injected intravenously into rabbits in a dose corresponding to

0·01 to 0·1 mgm. of moist bacilli, or 10 mgm. subcutaneously. Animals which have survived for two months are killed at the end of that time. In the case of *bovine* strains, death usually occurs earlier; *post-mortem* an acute generalised infection is found, the lungs especially showing abundant tubercles. *Human* strains commonly fail to kill rabbits within two months; when examined at this time they show only scanty isolated lesions in the internal organs (lungs and kidneys). As a rule, dysgonic growth on glycerol egg medium corresponds with high virulence for the rabbit characteristic of bovine strains, whereas eugonic cultures show the low virulence of human strains. Occasionally, however, there is a lack of correspondence between virulence for the rabbit and cultural characters *Avian* strains may be recognised by their appearance in cultures (moist luxuriant growth) together with their pathogenic action when fed to fowls and lack of virulence for the guinea-pig (*vide* p. 426).

Bacillus of Johne's Disease.—An acid-fast bacillus of considerable interest is that of Johne's Disease (*Mycobacterium paratuberculosis*), or the bacillus of "chronic bovine pseudo-tuberculous enteritis," the lesions produced by it being corrugated thickenings of the mucous membrane, especially of the small intestine. The disease has now been observed in various countries, and has been found to be comparatively common in Britain. The bacilli occur in large numbers in the lesions, the cells being often packed with them, and can readily be found in scrapings from the surface. They resemble the tubercle bacillus in appearance, but are distinctly shorter than the human type; they are equally acid-fast (Fig. 82). The organism was first cultivated by Twort and Ingram on egg medium to which there is added ½–1 per cent. of dried and powdered acid-fast bacilli, the Timothy-grass bacillus being most suitable; growth is slow, the colonies appearing after about four weeks in the primary cultures. It has been shown that plant-extracts may also contain the growth-promoting factor which this organism requires. "Johnin" prepared from cultures of the organism in the same way as tuberculin has been used as a specific diagnostic agent, but the specificity of the reaction with this product has been questioned. Sheep also contract the disease naturally.

CHAPTER XI

THE LEPROSY BACILLUS

LEPROSY is a disease of great interest, alike in its clinical and pathological aspects; whilst from the bacteriological point of view, also, it presents some striking peculiarities. The disease has a very wide geographical distribution. It occurs in certain parts of Europe—*e.g.* Norway, Russia, Greece—but is commonest in Asia, occurring in India, Syria, Persia, China, etc. It is prevalent in Africa, in the Pacific Islands, in certain parts of North and South America. In all these various regions the disease presents the same general features, and the study of its pathological and bacteriological characters, wherever such has been carried on, has yielded similar results.

Pathological Changes in Leprosy.—The disease is essentially chronic, a great amount of tissue change occurring without there being necessarily impairment of the general health. In other words, the local effects of the bacilli are well marked, often extreme, whilst the toxic phenomena are proportionately at a minimum unless during exacerbations.

There are two chief forms of leprosy. The one, usually called the nodular or "tubercular" form—*lepra tuberosa* or *tuberculosa*—is characterised by the growth of granulation tissue in a nodular form, or as a diffuse infiltration in the skin, in mucous membranes, etc., great disfigurement often resulting. In the other form, the anæsthetic—*maculo-anæsthetic* of Hansen and Looft—the outstanding changes are in the nerves, with consequent anæsthesia, paralysis of muscles, and trophic disturbances.

In the *nodular* form, the disease usually starts with the appearance of erythematous patches attended by fever, and these are followed by the development of small nodular thickenings in the skin, especially of the face, of the backs of hands and feet, and of the extensor aspects of arms and legs. These nodules enlarge and produce great distortion of the surface, so that, in the case of the face, an appearance is produced which has been described as "leonine." The thickenings occur chiefly in the cutis (Fig. 83), to a less extent in the subcutaneous tissue. The

epithelium often becomes stretched over them, and an oozing surface then develops, or actual ulceration may occur. The cornea and other parts of the eye, the mucous membrane of the mouth, nose, pharynx, and larynx may be the seat of similar nodular growths. Nodules in mucous membranes readily ulcerate, and in the nasal secretion large numbers of leprosy bacilli may be demonstrated; this is utilised for diagnostic purposes (*vide infra*). Internal organs, especially the spleen, liver, and testicles, may become secondarily affected. In

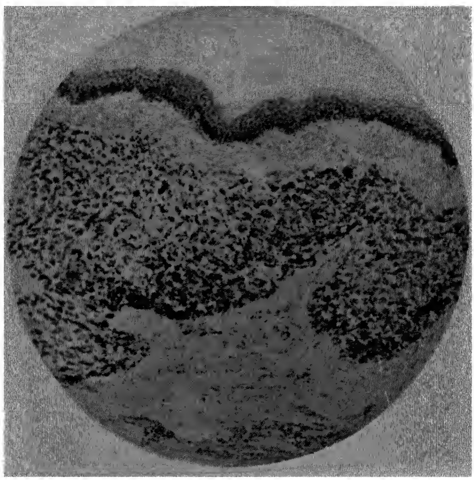


Fig. 83.—Section through leprous skin, showing the masses of cellular granulation tissue in the cutis; the dark points are cells containing bacilli deeply stained. See also Plate II, Fig. 8. Ziehl-Neelsen stain. ×80.

all situations the change is of the same nature, consisting in an abundant formation of granulation tissue, nodular or diffuse in its arrangement. In this tissue a large proportion of the cells are of rounded or oval shape, like hyaline leucocytes; a number of these may be of comparatively large size, and may show vacuolation of their protoplasm and a vesicular type of nucleus. These are often known as "lepra cells." Amongst the cellular elements there is a varying amount of stroma, which in the earlier lesions is scanty and delicate, but in the older lesions may be very dense. Periarteritis is a common change, and very frequently the superficial nerves become involved in the nodules,

and undergo atrophy. The tissue in the leprous lesions is comparatively vascular, at least when young, but, unlike tuberculous lesions, never shows caseation. Some of the lepra cells may contain several nuclei, but cells are not met with resembling in their appearance tubercle giant-cells, nor does a focal arrangement like that in tubercle follicles occur.

In the *maculo-anæsthetic* form, the lesion of the nerves is the outstanding feature. These are the seat of diffuse infiltrations, which lead to the destruction of the nerve fibres. In the earlier stages, in which the chief symptoms are pains along the nerves, there occur patches on the skin (*maculæ*), often of considerable size, the margins of which show a somewhat livid congestion. Later, these patches become pale in the central parts, and the periphery becomes pigmented. There then follows a remarkable series of trophic disturbances, in which the skin, muscles, and bones are especially involved. The skin often becomes atrophied, parchment-like, and anæsthetic ; frequently pemphigoid bullæ or other skin eruptions occur. Partly owing to injury to which the feet and hands are liable from their anæsthetic condition, and partly owing to trophic disturbances, necrosis and separation of parts are liable to occur. In this way great distortion or mutilation results. The lesions in the nerves are of the same nature as those described above, but the granulation tissue is scantier, and has a greater tendency to undergo cicatricial contraction. This is to be associated with the fact that the bacilli are present in fewer numbers.

Bacillus of Leprosy (*Mycobacterium lepræ*).—This bacillus was first observed in leprous tissues by Hansen in 1871, and was the subject of several communications by him in 1874 and later. Further researches, first by Neisser in 1879, and afterwards by observers in various parts of the world, agreed in their main results, and confirmed the accuracy of Hansen's observations. The bacilli, as seen in scrapings of ulcerated leprous nodules, or in sections, have the following characters. They are slender rods of practically the same size as tubercle bacilli, which they also resemble both in appearance and in staining reaction. They are straight or slightly curved, and usually occur in groups, though single forms and pairs may be noted (Fig. 84). When stained, they may have a uniform appearance, or the protoplasm may be beaded or granular like the tubercle bacillus. The beading is often of a " coarse " type. They often appear tapered at one or both extremities ; occasionally there is slight club-like swelling. Degenerated and partially broken-down forms are also seen. They take up the basic aniline stains more readily

than tubercle bacilli, but in order to stain them deeply, a powerful stain, such as carbol-fuchsin, is necessary. When stained, they resist decolorising, though they are more easily decolorised than tubercle bacilli (p. 121); variations, however, exist in this respect, some bacilli losing the stain more readily than others. The bacilli are also readily stained by Gram's method, and in this respect differ from the tubercle bacillus. They are regarded as non-motile and non-sporing organisms. Free granular

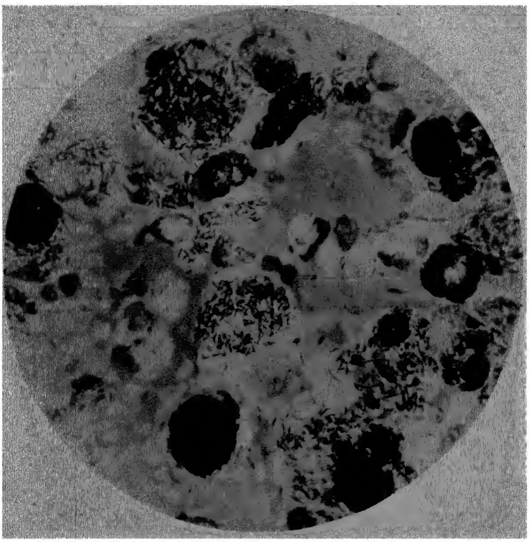


FIG. 84.—Leprosy bacilli in smear from nodule in skin, showing the arrangement of the bacilli within granulation tissue cells.
Ziehl-Neelsen stain. ×625.

forms of the leprosy bacillus have been observed and regarded as a definite phase in the life history of the organism (see tubercle bacillus, p. 409).

Distribution of Bacilli.—They occur in enormous numbers in the leprous lesions, especially in the nodular form—in fact, so numerous are they that the granulation tissue in sections, properly stained as above, presents quite a red colour under a low power of the microscope (Plate II., Fig. 8). The bacilli occur for the most part within the protoplasm of the round cells of the granulation tissue, and are often so numerous that the structure of the cells is quite obscured (Fig. 84). They

are often arranged in bundles which contain several bacilli lying parallel to one another, though the bundles lie in various directions (Plate II., Fig. 9). The appearance thus presented by the cells filled with bacilli is very characteristic. Bacilli are also found free in the lymphatic spaces, but the greater number are undoubtedly contained within the cells. They are also found in spindle-shaped connective-tissue cells, in endothelial cells, and in the walls of blood vessels. They have been observed in a venous thrombus. They are for the most part confined to the connective tissue, but a few may be seen in the hair follicles and glands of the skin. Occasionally one or two may be found in the surface epithelium, where they probably have been carried by leucocytes, but this position is, on the whole, exceptional They occur also in large numbers in the lymphatic glands associated with the affected parts. In the internal organs—liver, spleen, etc.—when leprous lesions are present, the bacilli are also found, though in relatively smaller numbers. In the nerves in the anæsthetic form they are less numerous than in the nodular lesions, and in the sclerosed parts it may be impossible to find any. They are absent from the *maculæ*, which are essentially trophic lesions.

Their spread is chiefly by the lymphatics, though distribution by the blood stream also occurs. They are said to have been found in the blood during the presence of fever and the eruption of fresh nodules, and they have also been observed in the blood vessels *post mortem*, chiefly contained within leucocytes. A few may be detected in some cases in various organs which show no structural change, especially in the capillaries. The brain and spinal cord are almost exempt, but in some cases bacilli have been found even within nerve cells.

Cultivation.—Attempts to cultivate the bacillus have in the majority of cases led to negative results. Further, it seems not unlikely that many of the acid-fast bacilli "cultivated" from leprosy represent saprophytic non-pathogenic organisms which are widely distributed and may occur on the skin. We have examined a number of the reputed *B. lepræ* strains, and, like others, have found them to resemble closely these non-pathogenic types in their biological characters. Kedrowski cultivated an organism which in culture appeared as a non-acid-fast diphtheroid, but which became acid-fast in the tissues of animals. When injected into mice and rats it produced, in a certain proportion of cases, lesions which presented the essential features of human leprosy, the bacilli occurring in large numbers within phagocytic cells. This organism grew very slowly and produced an irregular whitish growth of moist appearance closely resembling that of the avian tubercle bacillus. Bayon confirmed Kedrowski's results. Clegg grew a small acid-fast bacillus on plain agar medium along with amœbæ and symbiotic

bacteria, and then, by killing the contaminating organisms by means of heat, obtained a pure growth of a chromogenic acid-fast bacillus. Duval, following up this work, obtained confirmatory results, but in addition to Clegg's bacillus he cultivated a slowly growing non-chromogenic bacillus which only grew on special media. This latter he believed to be probably the causal organism. He laid stress especially upon its very slow growth, the colonies after eight to ten months being about the size of those of the influenza bacillus, and upon its requiring the presence of the products of protein digestion. Twort also claimed to have cultivated the organism on glycerol egg medium containing dead tubercle bacilli in the proportion of 1 per cent. Rost and Williams cultivated a pleomorphic organism, a streptothrix, which appeared in the form of bacilli or branched filaments, and both of these forms were acid-fast or non-acid-fast. Their organism, it is to be noted, however, grew comparatively rapidly, growth being visible within a week, while in the case of the organisms of Kedrowski, Duval, and Twort, growth only appeared after several weeks. Furthermore, the organisms of Duval and Twort appeared only in the bacillary form, whilst those of the other observers mentioned showed pleomorphism. Bayon compared the pathogenic properties of the bacilli cultivated by different workers, and found that only Kedrowski's bacillus and that cultivated by himself, which he regarded as the same organism, produced in animals lesions similar to those of leprosy, the cells in the lesions being packed with bacilli, and there appears to be no doubt that in his preparations a multiplication of the organism had taken place. It is quite clear that certain of the organisms cultivated by various workers and claimed to be leprosy bacilli present essential differences, and some of them certainly correspond closely to saprophytic acid-fast bacillary types (*vide* p. 428).

The successful cultivation of the leprosy bacillus has been claimed by Shiga. Leprosy nodules were triturated in a mortar with 5 per cent. sulphuric acid, and the emulsion was then centrifuged after standing for twenty minutes in the incubator. The deposit was washed with saline solution and again separated in the centrifuge. From it inoculations were made on a potato medium which had been previously boiled in 4 per cent. glycerol broth. After a month the bacilli in the inoculum had undergone degeneration; but after two months, though no visible growth was observed, they had multiplied and formed masses of well-stained organisms. By subculturing on glycerol agar small visible colonies were obtained.

An acid-fast bacillus has recently been isolated from leprosy nodules by McKinley and Soule. The cultures were obtained by inoculating a variety of media (egg media, glycerol potato, blood agar, etc.) and placing the cultures during incubation in an atmosphere consisting of 40 per cent. oxygen and 10 per cent. carbon dioxide. The growth developed slowly, small colonies being noticeable only after six weeks. The growths were non-chromogenic and represented acid-fast bacilli corresponding morphologically to the leprosy bacillus. Monkeys were inoculated intracutaneously with cultures in the supra-orbital region and developed a granulomatous lesion similar to that produced by direct inoculation of material from a case (*vide infra*). McKinley and Verder later used a medium of minced chick embryo in Tyrode's solution. Growth resulted in a few days and serial subcultivation also proved

successful. Salle has also grown the bacillus from leprosy lesions in chicken tissue cultures and obtained subcultures in minced chick embryo medium. The cultures showed both acid-fast and non-acid-fast organisms, the former being specially abundant when the tissue culture was in active growth. On transferring to ordinary media only the non-acid-fast forms were seen, although on re-transferring to tissue medium acid-fast forms reappeared.

Attempts to transmit leprosy to the lower animals have in general been unsuccessful. Sugai has stated that the Japanese dancing-mouse is comparatively susceptible to inoculation with leprous material, and Duval has confirmed this observation. The experiments of Kedrowski and Bayon have already been referred to. It is to be noted, however, that in all these cases success was only obtained in a certain proportion, and further that the picture of cells packed with bacilli has also been obtained by the injection of acid-fast saprophytes. In tuberculosis in mice a similar picture is obtained. It would accordingly be a mistake to place much reliance on this point. Experiments have also been performed on monkeys by Nicolle and Blaizot, Reenstierna and others, with inconclusive results. Recently McKinley and Soule have produced granulomatous nodules resembling leprosy lesions by the intracutaneous injection of material from human cases into the supra-orbital region of young monkeys (*e.g. Macacus rhesus*). It would appear that a localised lesion may be produced in monkeys by inoculation with material from human leprosy. This lesion, however, resolves, and there is no spread of the infection.

It is interesting to note that a disease occurs under natural conditions in rats which presents many points of close similarity to leprosy. It is very widespread, having been observed in Europe, Asia, America, and Australia; an excellent description was given by G. Dean. In this affection there are lesions in the skin which resemble those in leprosy, and the cells contain enormous numbers of an acid-fast bacillus. The disease can be transmitted to rats by inoculation with tissue material containing the bacilli, but not to animals of other species. The relations of this affection to human leprosy have not yet been established. Bayon claimed to have cultivated the bacillus of rat leprosy, and found that it was practically identical, as regards both cultural characters and pathogenic effects, with the organism obtained from the human disease. It is doubtful, however, whether the organism isolated was the true causal agent.

It would also appear that leprosy is not readily inoculable in the human subject. In a well-known case described by

Arning, a criminal in the Sandwich Islands was inoculated with leprosy tissue. Two or three years later, well-marked nodular leprosy appeared, and led to a fatal result. But this experiment is open to the objection that the individual before inoculation had been exposed to infection in a natural way, having been frequently in contact with lepers. Marchoux, however, has recorded the case of a young medical man in Paris whose skin was accidentally pricked by a needle while he was assisting at the operative removal of a skin nodule from a leper. About eight years later he developed leprous lesions. In other cases, inoculation experiments on healthy subjects and inoculations in other parts of leprous individuals have given negative results. It has been supposed by some that the failure to obtain cultures and to reproduce the disease experimentally may be partly due to the bacilli in the tissues being dead. According to de Langen also, attempts to superinfect the skin of lepers with material from lesions failed except in some cases where the inocula were derived from patients at the stage of acute exacerbation with fever.

The facts stated with regard to cultivation and inoculation experiments serve to distinguish the leprosy bacillus all the more strongly from other organisms. It was supposed at one time that leprosy was a form of tubercle, or tubercle modified in some way, but for this there is no evidence. It should also be mentioned that tubercle is a not uncommon complication in leprous subjects, in which case it presents the ordinary characters. It has been found that a considerable proportion of lepers react to tuberculin like tuberculous patients. This result has been variously interpreted, some considering that tuberculosis is also present in such cases, while others maintain that the reaction may be given in the absence of tubercle. If, as is probable, the latter is the case, the result most likely depends on the close relationship of the organisms of the two diseases ; it by no means proves their identity. Another curious fact is that the Wassermann reaction (p. 677) may be given by the serum of leprous patients (in about 50 per cent., according to some observers). It is doubtful whether the reaction in leprosy is independent of the concurrent presence of syphilis, as has been suggested by some observers. In a series of cases of leprosy in Malaya Amies found 28 per cent. gave positive Wassermann and Kahn reactions but considered this was not substantially in excess of the syphilis rate in the general population. Further, antisyphilitic treatment caused the reactions to become negative in a large proportion of cases. No method of treatment of

leprosy has been supplied from the bacteriological side, but the injection of chaulmoogra oil has been found to exert a favourable therapeutic effect, and has apparently led to cure in early cases. The ethyl esters to which the effect of the oil is attributed have been investigated by Rogers and have taken the place of the crude oil.

The mode by which leprosy is transmitted has been the subject of great controversy. It was at one time considered to be a hereditary disease transmitted from a parent to the offspring. There appears to be no doubt, however, that leprous subjects may bear children free from leprosy, and that healthy individuals entering a leprous district may contract the disease. Of the latter occurrence there is the well-known instance of Father Damien, who contracted leprosy after going to the Sandwich Islands. In view of all the facts, there can be little doubt that leprosy is transmitted by direct contact in certain conditions, though its contagiousness is not of a high order. Close contact over a long period, such as occurs in families, is necessary for the spread of the disease. It has been suggested also that certain insects may be responsible for transmission of the disease, but there is no reliable evidence to support the idea.

Methods of Diagnosis.—Film preparations should be made from the discharge of any ulcerated nodule which may be present, or from the scraping of a portion of excised tissue, and should be stained as above described. The presence of large numbers of bacilli situated within the cells and giving the staining reaction of leprosy bacilli, is conclusive. It is more satisfactory, however, to make microscopic sections through a portion of the excised tissue, when the structure of the nodule and the arrangement of the bacilli can be readily studied. Films of the nasal secretion (*vide supra*) are also examined in the same way, and may yield positive results even in maculo-anæsthetic cases. The points of difference between leprosy and tubercle have already been stated, and in most cases there is really no difficulty in distinguishing the two conditions. A negative result, on inoculating a guinea-pig with the suspected material, will exclude tuberculosis.

CHAPTER XII

THE BACILLI OF GLANDERS AND MELIOIDOSIS

Glanders Bacillus

The bacillus of glanders, *B. mallei* (*Actinobacillus mallei*), was discovered by Löffler and Schutz, the announcement of this discovery being made towards the end of 1882. They not only obtained pure cultures of this organism from the tissues in the disease, but by experiments on horses and other animals conclusively established its causal relationship. These have been fully confirmed. The same organism was also cultivated from the disease in the human subject, first by Weichselbaum in 1885, who obtained it from the pustules in a case of acute glanders in a woman, and by inoculation of animals obtained results similar to those of Löffler and Schutz.

The Natural Disease.—Glanders chiefly affects equines—horses, mules and asses. Horned cattle and pigs, on the other hand, are quite immune, while goats and sheep occupy an intermediate position, the former being rather more susceptible and occasionally suffering from the natural disease, as do also camels. Glanders occurs in some of the carnivora—cats, lions and tigers in menageries becoming infected from feeding on the carcases of animals affected with the disease. Many of the small rodents are highly susceptible to experimental inoculation (*vide infra*); in this connection it is of interest that infection with the allied *B. whitmori* occurs naturally in these animals. While prevalent at one time in this country, particularly among horses, glanders has now become a very rare disease, as a result of early diagnosis by means of mallein and slaughter of infected animals.

Glanders may occur also in man as the result of direct inoculation of some wound of the skin or other part by means of the discharges or diseased tissues of an affected animal, and hence has been commonest among grooms and others whose work brings them into contact with horses; even among them it is an exceedingly rare disease.

In horses the lesions are of two types, to which the names "glanders" proper and "farcy" have been given, though both may exist together. In glanders proper, the septum nasi and adjacent parts are chiefly affected, there occurring in the mucous membrane nodules which are at first firm and of somewhat translucent grey appearance. The growth of these is usually attended by inflammatory swelling and profuse catarrhal discharge. Afterwards the nodules soften in the centre, break down, and give rise to irregular ulcerations. Similar lesions, though in less degree, may be found in the respiratory passages Associated with these lesions there is usually implication of the lymphatic glands in the neck, mediastinum, etc. ; and there may be in the lungs, spleen, liver, etc., nodules of the size of a pea or larger, of greyish or yellow tint, firm or somewhat softened in the centre, and often surrounded by a congested zone. The term "farcy" is applied to the affection of the superficial lymphatic vessels and glands, which is specially seen where infection takes place through an abrasion of the skin, such as is often produced by the rubbing of the harness. The lymphatic vessels become irregularly thickened, so as to appear like knotted cords, and the associated lymphatic glands become enlarged and firm ; suppurative softening usually follows, and there may be ulceration. These thickenings are often spoken of as "farcy buds" and "farcy pipes." In farcy, also, secondary nodules may occur in internal organs and the nasal mucous membrane. The disease is often present in a "latent form," and its presence can only be detected by the mallein test (*vide* p. 462).

In man the disease is met with in two forms, an acute and a chronic—though intermediate forms also occur, and chronic cases may take on the characters of the acute disease. The site of inoculation is usually on the hand or arm—by means of some scratch or abrasion, or possibly by infection along a hair follicle—sometimes on the face, and occasionally on the mucous membrane of the mouth, nose, or eye. In the *acute* form there appears at the site of inoculation inflammatory swelling, attended usually with spreading redness, and the lymphatics in relation to the part also become inflamed, the appearances being those of a "poisoned wound." These local changes are soon followed by marked constitutional disturbance, and by a local or widespread eruption on the surface of the body, at first papular and afterwards pustular, and later there may form in the subcutaneous tissue and muscles larger masses which soften and suppurate, the pus being often mixed with blood ; suppuration may occur also in the joints. In some cases the nasal mucous membrane may be secondarily infected, and thence inflammatory swelling may spread to the tissues of the face. The patient usually dies in two or three weeks, sometimes sooner, with the symptoms of pyæmia. In addition to the lesions mentioned, there may be foci, usually suppurative, in the lungs (attended often with

pneumonic consolidation), in the spleen, liver, bone-marrow, salivary glands, etc. In the *chronic* form a local granulomatous condition may occur, which usually breaks down and gives rise to the formation of an irregular ulcer with thickened margins, and sanious, often foul, discharge. The ulceration spreads deeply as well as superficially, and the thickened lymphatics also have a great tendency to ulcerate, though the lymphatic system is not so prominently affected as in the horse. Deposits may form in the subcutaneous tissue and muscles, and the mucous membrane may become affected. The disease may run a very chronic course, lasting for months or even years, and recovery may occur; on the other hand, such a case may at any time take on the characters of the acute form of the disease and rapidly become fatal. Even when there is apparent recovery recurrence may occur.

FIG. 85.—Glanders bacilli—several contained within leucocytes—from peritoneal exudate in a guinea-pig.
Stained with weak carbol-fuchsin. ×1000.

The Glanders Bacillus.—*Microscopical Characters.*—The bacilli are minute rods, straight or slightly curved, with rounded ends (Fig. 85), and about the same length as tubercle bacilli, but rather thicker (3 to 5 μ by 0·4 μ). They show, however, considerable variations in size and in appearance, and their protoplasm is often broken up into a number of deeply stained portions with unstained intervals between. These characters are seen both in the tissues and in cultures, but, as in the case of many organisms, irregularities in form and size are more pronounced in cultures (Fig. 86). Filamentous forms 8 to 12 μ in length are sometimes met with. The organism is non-motile and does not form spores.

In the tissues the bacilli usually occur irregularly scattered among the cellular elements; a few may be contained within leucocytes and connective tissue cells, but the position of most is extracellular. They are most abundant in the acute

lesions, in which they may be found in considerable numbers; but in the chronic nodules, especially when softening has taken place, they are few in number, and it may be impossible to find any in sections.

Staining.—The glanders bacillus stains with simple watery solutions of the basic stains, but somewhat faintly (better when an alkali or a mordant, such as carbolic acid, is added), and even if deeply stained it readily loses the colour when a decolorising agent such as alcohol is applied. It is Gram-negative. In film preparations from fresh glanders nodules the bacilli can be readily found by staining with any of the ordinary combinations, *e.g.* carbol-thionin or weak carbol-fuchsin. In the case of sections, we have obtained the best results with carbol-thionin and dehydrating by the aniline-xylol method (p. 112).

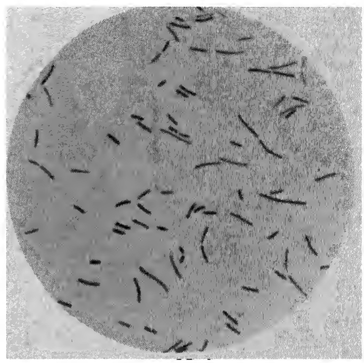


FIG. 86.—Glanders bacilli, from a pure culture on glycerol agar.
Stained with carbol-fuchsin and partially decolorised to show segmentation of protoplasm. ×1000.

McFadyean recommends that after sections have been stained in Löffler's methylene-blue and slightly decolorised in weak acetic acid, they should be treated for fifteen minutes with a saturated solution of tannic acid; thereafter they are washed thoroughly in water, and as a contrast-stain a 1 per cent. solution of acid fuchsin may be applied for half a minute; they are then dehydrated, cleared, and mounted.

Cultivation.—(For the methods of separation, *vide infra.*) The glanders bacillus grows readily on most of the ordinary media, growth taking place most rapidly at 35°–37° C. Though a certain amount of growth occurs down to 21° C., a temperature above 25° C. is always desirable.

On *agar* and *glycerol agar,* in stroke cultures, growth appears along the line as a uniform streak of greyish-white colour and somewhat transparent appearance, with moist-looking surface, and when touched with a wire is found to be of rather slimy

consistence. Later it spreads laterally for some distance, and the layer becomes of slightly brownish tint.

On *serum media* the growth is somewhat similar but more transparent, the separate colonies being in the form of round and almost clear drops. In subcultures at the body temperature growth is visible within twenty-four hours, but when fresh cultures are made from the tissues there may be no visible growth for two or three days. Serum media are much more suitable for cultivating from the tissues than the agar media.

In *broth*, growth forms at first a uniform turbidity, but soon settles to the bottom, and after a few days forms a fairly thick flocculent deposit of slimy and somewhat tenacious consistence.

On *potato* (provided the reaction is not too acid), at 30°–37° C. the glanders bacillus flourishes well and produces a characteristic appearance, incubation at the higher temperature, however, being advisable. Growth proceeds rapidly, and on the third day has usually formed a transparent layer of slightly yellowish tint, like clear honey in appearance. On subsequent days the growth still extends and becomes darker in colour and more opaque, till about the eighth day it has a reddish-brown or chocolate tint. The characters of the growth on potato, along with the microscopical appearances, serve to distinguish the glanders bacillus from other organisms. (*B. melitensis*, *B. abortus*, the cholera vibrio, and *B. pyocyaneus* produce a somewhat similar appearance, but they can be readily distinguished by their other characters.) Potato is also a suitable medium for starting cultures from the tissues; in this case minute transparent colonies become visible on the third day, and afterwards present the appearances just described.

Rough and smooth forms have been described. Fermentative properties are deficient. Acid formation from glucose has been found, but this is not constant.

Powers of Resistance.—The glanders bacillus is not killed at once by drying, but usually loses its vitality after fourteen days in the dry condition, though sometimes it lives longer. It is not quickly destroyed by putrefaction, as it has been found to be still active after remaining two or three weeks in putrefying fluids. It has comparatively feeble resistance to heat and antiseptics. Löffler found that it was killed in ten minutes in a fluid kept at 55° C., and in from two to three minutes by a 5 per cent. solution of carbolic acid.

Experimental Inoculation.—In horses, subcutaneous injection of the glanders bacillus in pure culture reproduces all the

essential features of the disease. This fact was established at a comparatively early date by Löffler and Schutz, who, after one doubtful experiment, successfully inoculated two horses in this way, the cultures used having been grown for several generations outside the body. The ass and mule are even more susceptible than the horse, the disease in the former running a more rapid course, but with similar lesions. The ass can be readily infected by simple scarification and inoculation with glanders secretion, etc. (Nocard). Infection by feeding is also successful.

Of small animals, field-mice and guinea-pigs are the most susceptible ; on the other hand, white mice are more resistant and after inoculation with pus containing *B. mallei* alone they live for five or six weeks (Sabolotny). In field-mice, subcutaneous inoculation is followed by a very rapid disease, usually leading to death within eight days, the organisms becoming generalised and producing numerous minute nodules, especially in the spleen, lungs, and liver. In the guinea-pig the disease is less acute. At the site of inoculation an inflammatory swelling forms, which soon softens and breaks down, leading to the formation of an irregular crateriform ulcer with indurated margins. The lymphatic vessels become infiltrated, and the corresponding lymphatic glands become enlarged to the size of peas or small nuts, softened, and semi-purulent. The animal sometimes dies in two or three weeks, sometimes not till later. Secondary nodules, in varying numbers in different cases, may be present in the spleen, lungs, bones, nasal mucous membrane, testicles, etc. ; in some cases a few nodules are found in the spleen alone. Intraperitoneal injection in the male guinea-pig is followed, as pointed out by Straus, by a very rapid and semi-purulent affection of the tunica vaginalis, shown during life by great swelling and redness of the testicles, which changes may be noticeable in two or three days, or even earlier. This method of inoculation has been found of service for purposes of cultivation and diagnosis ; however, strains of low virulence may fail to produce the characteristic results, while certain other organisms may cause the same lesions. Rabbits are less susceptible than guinea-pigs, and the effect of subcutaneous inoculation is somewhat uncertain. Accidental inoculation of the human subject with pure cultures of the bacillus has in more than one instance been followed by the acute form of the disease and a fatal result.

Action on the Tissues.—The glanders bacillus causes a more rapid and more marked inflammatory reaction than the tubercle bacillus ; there is more leucocytic infiltration and less proliferative

change. Thus the centre of an early glanders nodule shows a dense aggregation of leucocytes, most of which are polymorphonuclear, while in that region also many show fragmentation of nuclei with the formation of numerous chromatin granules. And further, the inflammatory change may be followed by suppurative softening of the tissue, especially in certain situations, such as the subcutaneous tissue and lymphatic glands. The nodules, therefore, in glanders, as Baumgarten put it, occupy an intermediate position between miliary abscesses and tubercles. The diffuse coagulative necrosis and caseation which are so common in tubercle do not occur to the same degree in glanders, and typical giant-cells are not formed. The tendency to spread by the lymphatics is always a well-marked feature, and when the bacilli gain entrance to the blood stream they soon settle in the various tissues and organs. Accordingly, even in acute cases it is usually quite impossible to detect the bacilli in the circulating blood, though sometimes they have been found. It is an interesting fact, shown by observations of the disease both in the human subject and in the horse, as well as by experiments on guinea-pigs, that the mucous membrane of the nose may become infected by means of the blood stream—another example of the tendency of organisms to settle in special sites. In infections with *B. mallei* supersensitiveness develops to products of the bacillus (" mallein ") similar to that of tuberculous subjects to tuberculin.

Mode of Spread.—Glanders usually spreads from a diseased animal by direct contagion with the discharge from the nose or from the sores, etc. There is no evidence that in man the disease is produced by inhalation of the bacilli in the dried condition. Some authorities consider that pulmonary glanders may be produced in this way in the horse, while others maintain that in all cases there is first a lesion of the nasal mucous membrane or of the skin surface, and that the lung is affected secondarily. Babés, however, found that the disease could be readily produced in susceptible animals by exposing them to an atmosphere in which cultures of the bacillus had been pulverised. He also found that inunction of the skin with vaseline containing the bacilli might produce the disease, the bacilli in this case entering along the hair follicles.

Mallein and its Preparation.—Mallein is obtained from cultures of the glanders bacillus and, like tuberculin, is really a mixture comprising (1) substances in the bodies of the bacilli, and (2) their soluble products, not destroyed by heat, along with substances derived from the culture medium. It is usually prepared from

cultures in glycerol broth. Such a culture, after being allowed to grow for three or four weeks, is sterilised by heat either in the autoclave at 115° C. or by steaming at 100° C. It is then filtered through paper. The filtrate constitutes dilute fluid mallein; it may then be concentrated to one-tenth of its volume by evaporation (crude mallein) or it may be preserved in the dilute form with the addition of 0·5 per cent. carbolic acid. When cool it keeps in the dark for months. Of dilute mallein (or of the concentrated product diluted 1 : 10) 1 c.c. is usually the dose for a horse (McFadyean). Foth has prepared a dry form of mallein by mixing the filtrate of a broth culture, evaporated to one-tenth of its bulk, with twenty-five or thirty times its volume of alcohol. A white precipitate is formed, which is dried over calcium chloride and then under diminished pressure. A dose of this dry mallein is 0·05 to 0·07 gram dissolved in sterile water before use.

The Use of Mallein as a Means of Diagnosis.—In using mallein for the diagnosis of glanders, the temperature of the animal ought to be observed for some hours beforehand, and after subcutaneous injection of a suitable dose, it is taken at definite intervals—according to McFadyean at the sixth, tenth, fourteenth, and eighteenth hours afterwards, and on the next day. Here both the local reaction and the temperature are of importance. In a glandered animal, at the site of inoculation there is a somewhat tender local swelling, which reaches a diameter of 5 inches at least, the maximum size not being attained until twenty-four hours afterwards. The temperature rises 1·5° to 2° C., or more, the maximum generally occurring in from eight to sixteen hours. If the temperature never rises as much as 1·5°, the reaction is considered doubtful. In the negative reaction given by an animal free from glanders, the rise of temperature does not usually exceed 1°, the local swelling reaches the diameter of 3 inches at most, and has much diminished at the end of twenty-four hours. In the case of dry mallein, local reaction is less marked. Also 0·2 c.c. crude mallein instilled into the conjunctival sac, gives in glandered animals a corresponding reaction to the ophthalmic tuberculin reaction in cases of tuberculosis (p. 434). Reactions may be obtained in glanders by cutaneous or intracutaneous applications of mallein. The intradermo-palpebral test has been much used; 0·1 c.c. of a 25 per cent. dilution in saline of crude mallein is injected intracutaneously close to the edge of the lower eyelid. A positive reaction consists in a firm, painful swelling of the lid with muco-purulent conjunctivitis, lasting for three to four days. In healthy animals there is at most slight œdema of the eye-lid. Veterinary authorities are practically unanimous as to the great value of the mallein test as a means of diagnosis. Animals at an advanced stage of the disease may fail to react.

Serum Reactions.—Shortly after the discovery of agglutination in typhoid fever, McFadyean found that the serum of infected horses possessed the power of agglutinating glanders bacilli, and this test has been extensively applied in the diagnosis of the disease. Normal serum may, however, agglutinate *B. mallei*; and Wilson has found titres over 1500 with sensitive strains. Accordingly natural agglutination must be excluded by careful quantitative tests. A titre of over 1000 is highly suggestive of the presence of glanders. The fixation of complement test is also applicable in the case of glanders,

and this has given valuable results in the hands of various observers. A precipitin reaction may be obtained on the addition of mallein or an extract of the glanders bacillus to the serum of a glandered animal. These reactions, which, of course, depend on the presence of antibodies in the blood in glanders, form important auxiliaries to the method of diagnosis by means of mallein. Different serological strains of *B. mallei* have been met with.

Methods of Examination.—Microscopic examination in a case of suspected glanders will at most reveal the presence of bacilli corresponding in their characters to the glanders bacillus. An absolute diagnosis cannot be made by this method. Cultures may be obtained by making successive strokes on coagulated serum or on potato, and incubating at 37° C. The colonies of the glanders bacillus do not appear till two or three days afterwards. This method may fail unless a considerable number of the glanders bacilli are present. The most certain method, however, is by inoculation of a guinea-pig. After intraperitoneal injection in a male guinea-pig the characteristic Straus reaction is rapidly produced. If, however, there have been other organisms present, the animal may die of a septic peritonitis, though even in such a case the glanders bacilli will be found to be more numerous in the tunica vaginalis, and may be cultivated from this situation. Where there is mixed infection, however, it is advisable first of all to inoculate a guinea-pig subcutaneously and then cultivate the organisms from the enlarged lymph glands. The application of mallein in diagnosis of the disease in the human subject is not justifiable. There is a certain risk that it may lead to the lesions assuming a more acute character; moreover, culture and inoculation tests are generally available. In the case of horses, etc., a diagnosis will, however, be much more easily and rapidly effected by means of mallein, or by one of the serum reactions described above. In some cases of acute glanders in the human subject the bacillus has been obtained in cultures from the blood during life.

The Bacillus of Melioidosis

This disease, which resembles glanders closely, was first observed by Whitmore in Rangoon, and its main features and the causal organism were described by him in 1913. He cultivated it from 38 cases and gave an account of its characters; the organism is now known as *B. whitmori* (*Actinobacillus pseudomallei*). The disease has also been found by Stanton and Fletcher to occur in the Malay States, and they have shown that it is naturally an epizootic among small rodents—rabbits, guinea-pigs, and rats; cats and dogs also are affected. It is believed that infection in man usually occurs by way of the alimentary tract, probably from ingestion of

food contaminated with the bacilli from the fæces of infected rodents. The clinical features of the disease in the human subject are very similar to those of glanders, though it tends to be, on the whole, rather more acute than the latter. The pathological changes likewise are of similar nature, a prominent feature being the occurrence of caseous or semi-purulent areas in the lungs with marked congestion around them; the histological changes also have been found to be practically identical in the two diseases. *B. whitmori* is a small bacillus which, in its morphological characters and in its staining reactions, resembles the *B. mallei*. It differs from the latter, however, in being actively motile, in growing on gelatin at 20° C. in which it produces liquefaction—as it does also on solidified serum—and in forming a pellicle in broth. The growth on glycerol agar is of two types, a corrugated—the commoner type—and a mucoid. The latter type produces on potato a growth similar to that of the glanders bacillus. In culture media, acid (but no gas) is formed slowly from glucose, lactose, and several other carbohydrates; indole is not produced. It is a fairly resistant organism outside the body. Susceptible animals can be infected by scarification, by feeding, or by the simple application of cultures to the nasal mucosa. A characteristic feature in the infected animals is a discharge from the nose and eyes, and *post mortem* numerous small nodules are found in the internal organs. With small doses of the organisms injected intraperitoneally in the guinea-pig the Straus reaction (p. 461) develops. Apparently the disease very rarely occurs in the horse, and Stanton and Fletcher have found that this animal is resistant to inoculation. These observers have found that *B. whitmori* is serologically closely allied to the *B. mallei*; in fact, a strain of the latter gave almost similar reactions. They found that a case of chronic human melioidosis gave a positive mallein reaction, and that the serum agglutinated certain strains of *B. mallei*. From this short account it will be seen that while melioidosis and glanders closely resemble one another, they present also interesting points of difference.

CHAPTER XIII

ACTINOMYCES (STREPTOTHRIX GROUP) AND ALLIED ORGANISMS

The term " actinomycosis " may conveniently be retained for infections caused by a number of allied species in which the parasite—variously called Actinomyces, Streptothrix, or Nocardia —forms " granules " or colonies composed of a mycelium or meshwork of a branched filamentous organism, which in certain cases exhibits radial club-shaped bodies. Actinomycosis occurs in man in common with certain of the domestic animals, though it is more frequent in the latter, especially in oxen, swine, and horses. The lesions are either suppurative or granulomatous —sometimes in the form of tubercle-like nodules—and often they partake of both characters; in cattle there is frequently great new formation of tissue. Bollinger first described the parasite in 1877, and the botanist Harz applied the name *Actinomyces* or *Ray fungus* owing to the radiate club-shaped structures characteristic of its colonies in the tissues. In 1878 Israel described the parasite in the human subject. It has been found by Lignières and Spitz also that in a common type of actinomycosis in the ox the colonies are formed not by a streptothrix but by a bacillus to which they have given the name of *Actinobacillus*. Madura disease also comes into this group though its etiology, geographical distribution, and pathological features distinguish it from actinomycosis. Other distinct species of streptothrix, without the characteristic arrangement, have also been cultivated from isolated cases of disease in the human subject where the lesions resembled more or less closely those of actinomycosis. In several instances the organism has been found to be " acid-fast," and the actinomyces group is evidently related through intermediate forms with the tubercle bacillus and the other acid-fast bacilli (*vide* p. 409).

Naked-Eye Characters of the Parasites.—The actinomyces grows in the tissues in the form of little round masses or colonies, which, when fully developed, are easily visible to the naked eye,

the largest being about 1 mm. in diameter, whilst all sizes below this may be found. When suppuration is present, the colonies lie free in the pus; when there is no suppuration, they are embedded in the granulation tissue, but are usually surrounded by a zone of softer tissue. They may be seen in the pus by spreading it out in a thin layer (Fig. 87). They may be transparent or jelly-like, or they may be opaque and of various colours—white, yellow, or greenish. The appearance depends upon their age and also upon their structure, the younger colonies being more or less transparent, the older ones being usually opaque. They are generally of soft, sometimes tallow-like, consistence, though sometimes in the ox they are gritty, owing to the presence of calcareous deposit. In the human subject they usually appear as small specks of semi-translucent appearance, and of greenish-grey tint, but they may be distinctly yellow. It must be noted that similar granules composed of masses of the parasite may occur in the tissues in widely different infections, *e.g.* botryomycosis due to staphylococcus in the horse, and sometimes after inoculation with the tubercle bacillus.

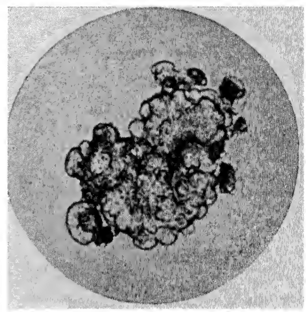


FIG. 87.—Low-power view of untreated colony of actinomyces in pus. ×20.

Microscopical Characters.—Three different morphological elements have been recognised, namely, filaments, spores or conidia, and clubs.

1. The *filaments* are comparatively thin, measuring about 0·6 to 1 μ in diameter, but they are often of considerable length. They are composed of a central protoplasm enclosed by a sheath. The latter, which is most easily made out in the older filaments with granular protoplasm, occasionally contains granules of pigment. In the centre of a colony developing in the tissues the filaments interlace with one another, and form an irregular network which may be loose or dense; at the periphery they are often arranged in a somewhat radiating manner, and run outwards in a wavy or even spiral course. They also show true branching, a character which at once distinguishes them from the ordinary bacteria. Between the filaments there is a finely granular or homogeneous ground

substance. Most of the colonies at an early stage are chiefly constituted by filaments loosely arranged (Fig. 88); but later, part of the growth may become so dense that its structure cannot be made out. This dense part, starting excentrically, may grow round the colony to form a hollow sphere, from the outer surface of which filaments radiate for a short distance. The filaments usually stain uniformly in the younger colonies and are Gram-positive, but often the staining by this method is irregular, portions of a filament staining violet, while other parts

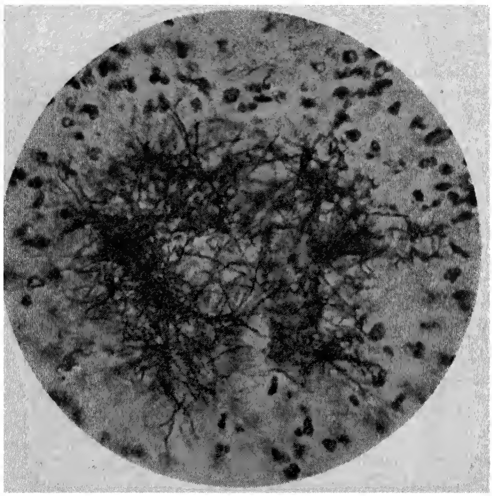


Fig. 88.—Actinomycosis of human liver, showing a colony of the parasite composed of a felted mass of filaments surrounded by pus. Paraffin section; stained by Gram's method and safranin. ×500.

are decolorised and take up the counter-stain; also a beaded appearance of the filaments may be observed. In the older colonies the filaments may be segmented so as to give the appearance of a chain of bacilli or of cocci, though the sheath enclosing them may generally be distinguished. Rod-shaped and spherical forms may also be seen lying free (Fig. 89). In culture some forms of actinomyces develop mainly as rod-shaped structures (resembling diphtheroid bacilli) with only a few branched and filamentous elements.

2. *Spores or Conidia*.—Some of the filaments of the actinomyces when growing on a culture medium become segmented into spherical forms (referred to above), and these structures

have been regarded as reproductive conidia and capable of forming new colonies on becoming free from the original growth. They have been reported to possess somewhat higher powers of resistance than the filaments, though less than the spores of most of the lower bacteria; cultures containing spores can resist a temperature from five to ten degrees higher than spore-free cultures (Foulerton). An exposure to 75° C. for half an hour is sufficient to kill most streptothrices or their spores. These supposed conidia are readily stained by Gram's method. It is questionable whether *Actinomyces bovis*, the common type of actinomyces found in human lesions, produces true conidial bodies.

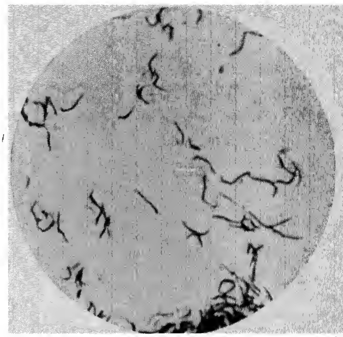


FIG. 89.—Film of crushed colony of actinomyces from a human case. Stained by Gram's method. ×1000.

3. *Clubs.*—These are elongated pear-shaped bodies which are seen at the periphery of the colony, and are formed by a sort of hyaline swelling of the sheath around the free extremity of a filament (Figs. 90, 91). They are usually homogeneous and structureless in appearance. In the human subject the clubs are often comparatively fragile structures, which are easily broken down, and may sometimes be dissolved in water. Sometimes they are well seen when examined in the fresh condition, but in hardened specimens are no longer distinguishable. In specimens stained by Gram's method they are usually not coloured by the violet, but take readily a contrast-stain; sometimes a darkly stained filament can be seen running for a distance in the centre, and may have a knob-like extremity. In many of the colonies in the human subject the clubs

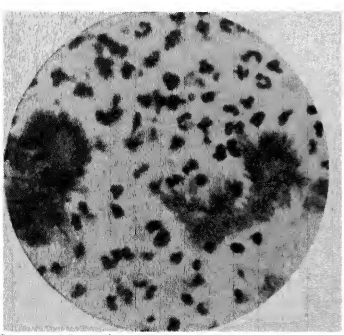


FIG. 90.—Actinomyces in human kidney, showing clubs radially arranged and surrounded by pus. The filaments had practically disappeared.
Paraffin section; stained with hæmatoxylin and fuchsin. ×500.

are absent. In the ox, on the other hand, where there are much older colonies, the clubs constitute the most prominent feature, and often form a dense fringe around the colony, staining by Gram's method. Occasionally in very chronic lesions in the human subject the clubs stain with Gram's method. Clubs showing intermediate staining reactions have been described in the ox by McFadyean. It is not clear whether the club formation represents a reaction on the

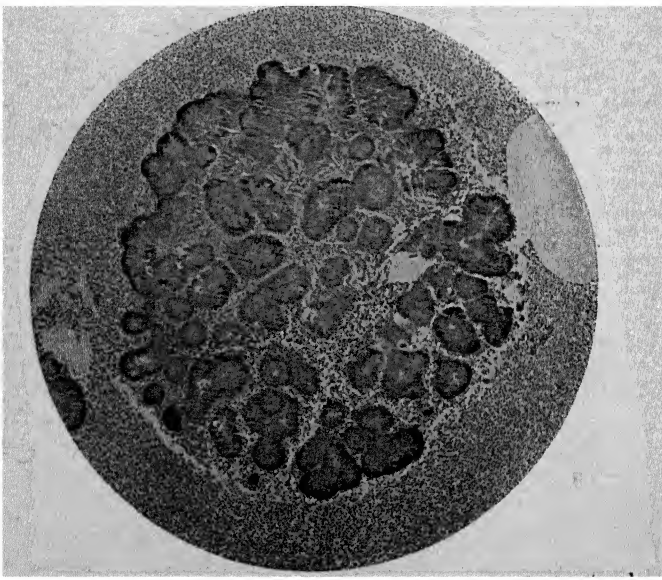


FIG. 91.—Colonies of actinomyces, showing general structural arrangement and clubs at periphery. From pus in human subject. Stained by Gram's method and safranin. ×60.

part of the parasite toward the tissues of the host and may be defensive, or whether it is degenerative in nature. In support of the latter view it has been stated that when dead organisms are introduced into the tissues club-formation may occur. In the majority of cases in the ox no filaments can be detected in the colonies, which are composed almost entirely of radially arranged clubs, and it is from such colonies that Lignières and Spitz have cultivated the *Actinobacillus* (*vide* p. 477).

Tissue Lesions.—In the human subject the lesions are of a

chronic inflammatory type, usually ending in a spreading suppuration. In some cases there is a comparatively large production of granulation tissue, with only a little softening in the centre, so that the mass feels solid. In most cases, however, and especially in internal organs, suppuration is the outstanding feature ; this is associated with abundant growth of the parasite in the filamentous form. In an organ such as the liver, multiple foci of suppuration are seen at the spreading margin of the lesion, often presenting a honeycomb appearance, while the colonies of the parasite may be seen in the pus with the naked eye. In the older parts the abscesses have become confluent, and formed large areas of suppuration.

In cattle the tissue reaction is more of a formative type, there being abundant growth of granulation tissue, which may result in large tumour-like masses, usually of more or less nodular character, and often consisting of well-developed fibrous tissue containing areas of younger formation, in which, however, irregular abscess formation may be present. The cells immediately around the colonies are usually irregularly rounded, or may even be somewhat columnar in shape, while farther out they become spindle-shaped and concentrically arranged. It is not uncommon to find leucocytes or granulation tissue invading the substance of the colonies, and portions of the parasite may be contained within leucocytes or within small giant-cells, which are sometimes present. A similar invasion of old colonies by leucocytes is sometimes seen in human actinomycosis. The disease usually remains quite local, or spreads by continuity. It may produce tumour-like masses in the region of the jaw or neck, or it may specially affect the palate or tongue, in the latter producing enlargement and induration, with nodular thickening on the surface—the condition known as " woody tongue."

Origin and Distribution of Lesions.—In the human subject the primary lesion is most commonly situated about the face or neck, this indicating that the entrance takes place in the mouth or throat—sometimes in the region of a decayed tooth, by the crypts of the tonsil, or by some abrasion of the mucous membrane. Swelling and suppuration may then follow in the vicinity and may spread in various directions, the bones often becoming affected. In a considerable number of cases the primary lesion is in some part of the intestine, generally the large intestine, and not infrequently in connection with the appendix. A peculiar affection of the intestine has been described, in which slightly raised plaques are found both in the large and small intestines, these plaques being composed almost exclusively of masses of the actinomyces along with epithelial cells. This, however, is a rare condition. The path of entrance may also be by the respiratory passages, the primary lesion being pulmonary or

peribronchial ; extensive suppuration in the lungs may result. Infection may, however, occur by the tongue, by the skin surface in any part of the body, and lastly, by the female genital tract, as in a case recorded by Grainger Stewart and Muir, in which both ovaries and both Fallopian tubes were affected.

When the parasite has invaded the tissues by any of these channels, secondary or "metastatic" abscesses may occur in internal organs. The liver is the organ most frequently affected, though abscesses may occur in the lungs, brain (where a primary meningitis may also occur), kidneys, etc. In such cases the spread takes place by the blood stream, and it is possible that leucocytes may be the carriers of the infection, as it is not uncommon to find leucocytes in the neighbourhood of a colony containing small portions of the filaments in their interior.

Source of the Infection.—At one time the view was held that the source of this organism was grain, and especially barley, on which it grew as a saprophyte. Both in the ox and in the pig the parasite has been found growing around fragments of grain, embedded in a mucous membrane. There are besides recorded in the human subject a certain number of cases in which there was a history of penetration of a mucous surface by a portion of grain ; and in certain cases such material has been found embedded in an infected area. It has been shown, however, that the type of streptothrix isolated from the majority of cases in man and cattle is a strict parasite, incapable of growing at a temperature much below that of the body, and requiring partial anaerobic conditions. In these respects it is unlike the common saprophytic actinomyces which in cultures grow readily at room temperature and are aerobic. There is no evidence that the disease is contagious in man, and it is usually impossible to trace the human infection to an animal source. This does not exclude the possibility of transmission from animals or infected persons. Carriers might also be responsible for spreading the disease. Infectivity must, however, be exceedingly slight. A certain amount of evidence has been collected to show that this organism may possibly occur as an inhabitant of the alimentary canal. It has been shown by Lord, for example, that mycelial organisms similar to actinomyces may be found in carious teeth and in the tonsillar crypts, and he has produced in animals inoculated intraperitoneally with these organisms, granulomatous lesions containing typical actinomyces colonies made up of mycelium and peripheral clubs. Tunnicliff has also described an actinomyces-like organism in tonsillar "granules." Penetration of a mucous

surface by a fragment of grain may act merely by facilitating the invasion of the organism and its establishment in the tissue.

Cultural Characters.—Cultures obtained by various investigators differ in essential particulars. The following classical types may be described :

Actinomyces bovis (Israel and Wolff).—The organism obtained in culture by Israel and Wolff is probably the same as that described in detail later by J. H. Wright, who obtained it in pure condition from fifteen cases of the disease. It is unable to grow freely under aerobic conditions, but is micro-aerophilic, *i.e.* flourishes best when the oxygen tension is reduced, or in an atmosphere of carbon dioxide. It grows best at 37° C. and ceases to grow at a temperature a little below that of the body. On the surface of agar under anaerobic conditions the organism produces dense rounded colonies of greyish-white colour, which sometimes assume a rosette form. A special feature of the growth was described by Wright, namely, that in a shake culture in glucose agar the colonies are most numerous and form a dense zone about half an inch from the surface of the medium, that is, where the conditions are suited for a micro-aerophilic organism. In broth, growth takes place at the bottom of the medium in rounded masses which afterwards undergo disintegration. When the organism is grown in the presence of serum or other animal fluids, the formation of true clubs occurs at the extremity of some of the filaments (Fig. 92B). Differences have been described in the appearance, colour, and other characters of cultures isolated by various workers. These variations may be due partly to differences in the conditions of cultivation, which are known to have a marked effect on the characters of a given strain. But it is most likely that a number of types are included under the name *Actinomyces bovis*.

Actinomyces bovis apparently does not occur in nature, but according to Naeslund the mouth is its chief habitat normally. Observations by workers in different countries clearly demonstrate that this organism is the predominant streptothrix type in human infections. Harbitz and Gröndahl in Norway obtained pure cultures from ten different cases, and in each instance the organism grew only under anaerobic conditions and presented the characters described above. They also obtained the same organism in culture from the disease in the ox. In this species it is the chief causal agent of actinomycosis in the bones of the head. Henry cultivated from actinomycotic meningitis an organism which was a strict anaerobe and which

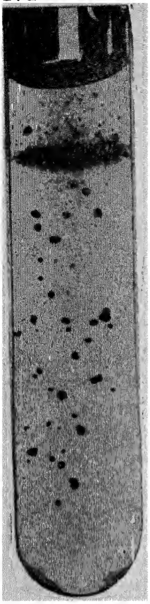


FIG. 92A.[1]—Shake culture of actinomyces in glucose agar, showing the maximum growth at some distance from the surface of the medium.

exhibited similar characters. More recently, Colebrook in this country has studied twenty-four strains of human origin. Of these, twenty-one conformed in their general characters and resembled the Israel and Wolff type, while two others showed slight differences in the characters of their growths. A considerable series of strains isolated by us from human cases have all conformed to the Israel and Wolff type.

Inoculation with the organism of Israel and Wolff in various animals, including guinea-pigs and rabbits, has given rise to granulomatous nodules, in which the characteristic colonies are present, though the lesions usually have not a progressive character. Magnusson, however, has obtained a nodule as large as a man's fist on inoculation of a cow. According to Naeslund's observations the presence of mixed infection plays an important part in conducing to the development of progressive actinomycosis.

Bacillus actinomycetem comitans.—It is of particular biological interest that associated with the mycelial organism in actinomycotic lesions there frequently occurs a minute non-motile Gram-negative coccoid or cocco-bacillary organism measuring often not more than 0·5 μ in its longest diameter and resembling in morph-

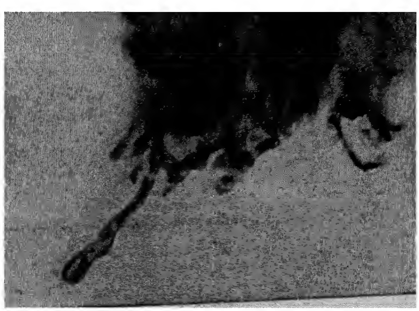


FIG. 92B.[1]—Section of a colony of actinomyces from a culture in blood serum, showing the formation of clubs at the periphery. ×1500.

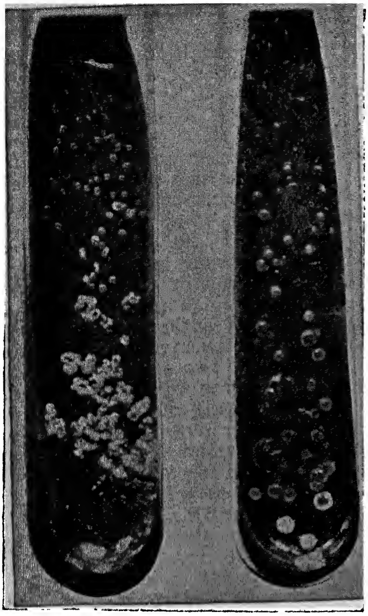
[1] For Figs. 92A and 92B we are indebted to Dr. J. Homer Wright of Boston, U.S.A.

ology the *Bacillus melitensis* (*q.v.*). This concomitant organism was first described in detail by Klinger, who named it *B. actinomycetem comitans*. Colebrook has observed it in the majority of the cases studied by him, and we have also noted it in a certain proportion of cases. This organism can be cultivated separately from the actinomyces, both aerobically and anaerobically, and growth occurs on ordinary media at 37° C. The colonies are small, discrete, semi-transparent whitish discs about 1 mm. in diameter when fully grown, and contrast in size and appearance with the colonies of the mycelial organism. In cultures from actinomycotic lesions the small colonies of the concomitant may often be seen in groups round the larger raised colonies of the actinomyces It is comparatively easy to separate this organism from the actinomyces by the usual methods of obtaining pure cultures, especially as it grows aerobically in contrast with the anaerobic growth of the common types of actinomyces, but it may be difficult to obtain pure growths of the mycelial organism free from the concomitant. In the case of two strains we have studied, it was found impossible to separate the mycelial organism from the concomitant, even after repeated plating and sub-culturing single isolated colonies. A growth of the latter invariably developed from the mycelial organism. One of these strains, however, when maintained in culture for two years was found to be ultimately free from the concomitant. It has been suggested that it may be a derivative or variant of the mycelial organism and not an independent species. On the other hand, its biological characters are so entirely different from the actinomyces that it might justifiably be regarded as a separate organism. Though possessing some biological similarity to the actinobacillus, it cannot be identified with this organism (*vide infra*). Inoculation of animals with pure cultures of this concomitant organism produces no specific effects. It is impossible at present to offer any satisfactory explanation of its occurrence.

Actinomyces graminis [1] (Boström).—On *agar* or *glycerol agar* at 37° C., growth is generally visible on the third or fourth day in the form of little transparent drops which gradually enlarge and form rounded projections of a reddish-yellow tint and somewhat transparent appearance, like drops of amber. The growths tend to remain separate, and even when they become confluent, the nodular character is maintained. They have a tough consistence, being with difficulty broken up, and adhere firmly to the surface of the agar. Older growths often show on the surface a sort of corrugated aspect, and may sometimes present the appearance of having been dusted with a brownish-yellow powder (Fig. 93).

In the cultures at an early stage the growth is composed of branching filaments, which stain uniformly (Fig. 94), but later some of the filaments may show segmentation into bacillary

[1] This name has been suggested by Topley and Wilson.



A B

Fig. 93.—Cultures of Streptothrix actino-myces (Boström) on glycerol agar, of about three weeks' growth. The growth in A is at places somewhat corrugated on the surface. Natural size.

and coccal structures, and superficial filaments may show formation of conidia, the latter accounting for the powdery appearance of old cultures. Slight bulbous thickenings may be seen at the end of some of the filaments, but true clubs have not been observed.

In *gelatin* the same tendency to grow in little spherical masses is seen, and the medium becomes very slowly liquefied. When this occurs, the liquefied portion has a brownish colour and somewhat syrupy consistence, and the growths may be seen at the bottom, as little balls, from the surface of which filaments radiate.

It occurs as a saprophyte on grain, and may occur in the mouth and throat of man and animals. Similar organisms are of very common occurrence in specimens of soil. Undoubted, though rare, infections of man with this organism have occurred in the lung (Biggart) and a similar case has recently been brought to our notice by Dr. W. B. Kyles, in which sputum, pleural exudate and abscesses in the thoracic wall were all swarming with the parasite; in the two latter situations it was in pure

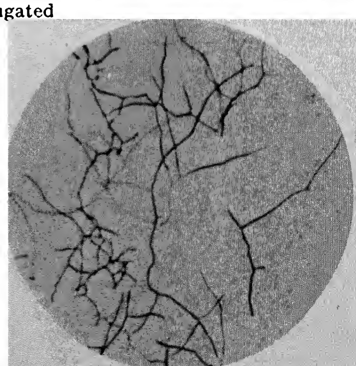


Fig. 94.—Actinomyces, from a culture on glycerol agar, showing branched filaments. See also Plate III., Fig. 10. Stained by Gram's method. ×1000.

culture. Inoculation experiments have given negative results, and it has been doubted whether this organism plays a causal role in actinomycosis.

Actinobacillus lignièresi.—This organism was cultivated by Lignières and Spitz from a number of cases of actinomycosis in the ox, in which no filaments could be detected in the granules. It grows readily on most ordinary media. It is a small bacillus, measuring about 1·5 μ in length and 0·4 μ in thickness, Gram-negative and non-motile. On agar it forms in the primary cultures rounded semi-transparent colonies which reach 1·5 mm. in diameter; in subcultures it forms a continuous layer of similar character. Subcutaneous injection in the sheep and ox, and intraperitoneal injection in the guinea-pig, give rise to lesions in which the characteristic granules with clubs are reproduced. These results have been substantially confirmed by F. Griffith and Magnusson. In twenty-three out of forty cases of bovine "actinomycosis," Griffith obtained a similar organism. He states that the majority of cases of bovine "actinomycosis" in this country are due to the actinobacillus. Its behaviour in the tissues differs from that of *Actinomyces bovis*; the latter is chiefly responsible for affections of bone and it does not involve the lymph glands. On the other hand, *Actinobacillus* affects the soft tissues almost exclusively, *e.g.* the tongue; it spreads to the lymphatics and it produces the form of actinomycosis which is benefited by iodide treatment. In pigs this organism causes actinomycosis of the udder—in a proportion of cases, however, staphylococcus is the causal organism. Infections with actinobacillus are exceedingly rare in the human subject (Beaver and Thompson). The term "actinobacillosis" might be more applicable to this condition.

Actinomyces actinoides.—This pleomorphic organism, which resembles actinobacillus in the tissues, was found in pure culture by Theobold Smith in the lesions in an epidemic of broncho-pneumonia in calves. To obtain cultures, *e.g.* on coagulated serum, an atmosphere rich in CO_2 is required.

A number of other Streptothrices have been recovered from cases of disease in man or animals. Certain of these are acid-fast. The following are representative forms.

Actinomyces asteroides.—A species of streptothrix was cultivated by Eppinger from a brain abscess, and called by him "Cladothrix asteroides," from the appearance of its colonies on culture media. A case of general streptothrix infection in the human subject described by Stuart McDonald was probably due to the same organism as Eppinger's. In the tissues it grows in a somewhat

diffuse manner, and does not form clubs; in rabbits and guinea-pigs it produces tubercle-like lesions. It grows readily on agar under aerobic conditions, forming branched filaments which tend to break up into short segments (Fig. 95). It is Gram-positive and especially in old cultures, weakly acid-fast. To the naked eye the growth is dry and at first whitish, later ochre yellow; on potato the culture becomes finally brick red.

Flexner observed a streptothrix in the lungs associated with lesions somewhat like a rapid phthisis, and applied the name "pseudo-tuberculosis hominis streptothricea"; an apparently similar condition has been described by Buchholz. Berestnew cultivated two species of streptothrix from suppurative lesions, one of which is acid-fast and grows only in anaerobic conditions. Birt and Leishman described another acid-fast streptothrix obtained from cirrhotic nodules in the lungs of a man. This organism grew readily on ordinary media, forming a white growth which was powdery owing to formation of conidia and which afterwards assumed a pinkish colour; it was pathogenic for guinea-pigs, in which it caused caseous lesions. Henrici and Gardner's *Actinomyces gypsoideus*, is similar.

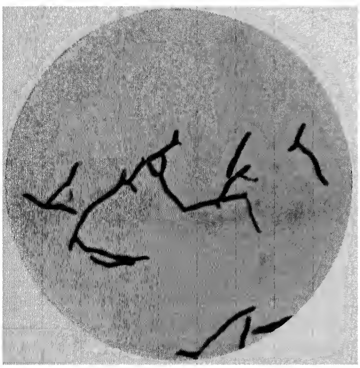


FIG. 95.—Eppinger's streptothrix from a forty-eight hours' culture on Löffler's serum.
Stained by Gram's method. ×1000.

Actinomyces farcinicus has been shown by Nocard to be the cause of a disease of the ox, "farcin du bœuf," in which there occur tumour-like masses of granulation tissue. It grows readily under aerobic conditions and is acid-fast.

Bacillus necrophorus (*Actinomyces necrophorus*).—A pleomorphous Gram-negative, non-acid-fast organism varying from coccoid forms to long, wavy, unbranching filaments arranged in felted masses, has been found in necrotic lesions in animals and man. Sometimes thickenings are present in the course of the filaments or at their ends, and staining with methylene-blue shows metachromatic beads. Its presence in pure culture in the lesions in certain cases indicates that it is the causal organism. It was described as the cause of "diphtheria of calves" by Bang, Schmorl and others. Also it produces abscesses in the liver and lung—"bacillary necrosis"—and gangrenous conditions of the skin; cattle especially are affected, but these conditions also occur in other domestic animals. Similar lesions have been recorded in man. Cultures are readily obtained in fluid media at 30° to 40° C., but only under anaerobic conditions: they have a characteristic unpleasant smell. On solid media containing serum the organism grows but with difficulty; the colonies have fimbriate borders. Rabbits and mice are very susceptible to inoculation and die with a marked local necrotic lesion. In addition to producing local effects, the organism also

secretes an exotoxin with general action. Beveridge, who has examined a series of cultures of animal origin, finds no evidence of strains specific for particular hosts.

B. funduliformis is a similar organism. This and related bacteria were found by Veillon and Zuber in suppurative conditions, *e g.* in the peritoneum associated with appendicitis. Beaver and others have observed abscesses in the liver in man in which it was present in pure culture. On inoculation of cultures into guinea-pigs and rabbits, abscesses are produced.

Organisms of Madura Disease

Madura disease or mycetoma resembles actinomycosis both as regards the general characters of the lesions and the occurrence of the parasite in the form of colonies or "granules." It most frequently affects the foot; hence the disease is often spoken of as "Madura foot." The hand is rarely affected. In the diseased parts there is a slow growth of granulation tissue which has an irregularly nodular character, and in the centre of the nodules there occurs purulent softening, which is often followed by the formation of fistulous openings and ulcers. There are great enlargement and distortion of the part and frequently caries and necrosis of the bones. Within the softened cavities and also in the spaces between the fibrous tissue, small rounded bodies or granules, consisting of colonies of the parasite, are present. These may have a yellowish or pinkish colour, compared from their appearance to fish roe, or they may be black like grains of gunpowder, and may by their conglomeration form nodules of considerable size. Hence a *pale variety* and a *black variety* of the disease have been distinguished; in both varieties the granules mentioned reach a rather larger size than in actinomycosis. The granules are present in the purulent secretion from the fistulous openings. This disease is comparatively common in India and in various other parts of the tropics; it has also been met with in Algiers and in America. Its course is of an extremely chronic nature, and though the local disease is incurable except by operation, the parasite never produces secondary lesions in internal organs. Each variety is caused by several different species of organisms. The pale variety is chiefly due to an actinomyces of which the description was originally given by Vincent. The black variety is caused by higher fungi.

Actinomyces maduræ (Vincent).—When the roe-like granules in the pale variety of mycetoma are examined microscopically they show in their interior an abundant mass of branching filaments with mycelial arrangement. There may also be

present at the periphery club-like structures; sometimes they are absent. These structures often have an elongated wedge-shape, forming an outer zone to the colony, and in some cases the filaments can be found to be connected with them. The colonies are often markedly degenerate, the mycelium may be densely matted and present an almost structureless appearance in the tissue. Growth takes place under aerobic conditions. Cultures on agar resemble that of Boström's actinomyces and consist of large, raised, knob-like colonies firmly adherent to the medium. The growth, however, develops a pink colour, which is apparently characteristic of the strains that have been isolated. In gelatin there is no liquefaction of the medium. The filaments which are long and branched, are Gram-positive, but not acid-fast. Experimental inoculation of various animals has failed to reproduce the disease.

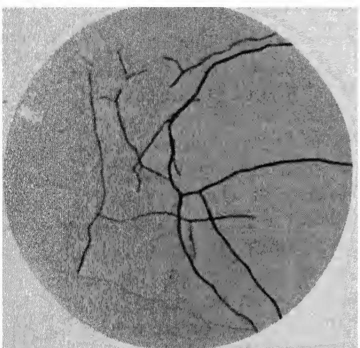


FIG. 96.—*Actinomyces maduræ*, showing branching filaments. From a culture on agar.
Stained by Gram's method. ×1000.

Hyphomycetes of Mycetoma.—An organism of this type was isolated by J. H. Wright from the black varieties of mycetoma, and has been obtained in pure cultures from the lesion. The pigment may be dissolved by soaking the granules for a few minutes in hypochlorite of sodium solution, and the granules may then be crushed out beneath a cover-glass and examined microscopically. The granules are composed of a somewhat homogeneous ground-substance impregnated with pigment, and in this there is a mycelium of thick filaments or hyphæ, many of the segments of which are swollen; at the periphery the hyphæ form a zone with radiate arrangement. In many of the older granules the parasite is largely degenerated and presents an amorphous appearance. Wright planted over sixty of the black granules in various culture media, and obtained cultures of a hyphomycete from about a third of these. The organism grows well on agar, broth, potato, etc.; on agar it forms a felted mass of greyisn colour, and in old cultures black

granules appear amongst the mycelium. Microscopically the parasite appears as a mycelium of thick branching filaments with delicate transverse septa ; in the older threads the segments become swollen, so that strings of oval-shaped bodies result. No signs of spore-formation have been noted. Inoculation of animals with cultures gave negative results, as did also direct inoculation with the black granules from the tissues. This organism is difficult to classify, but it is apparently related to fungi described by Brumpt and others in the black variety of mycetoma and grouped under the generic name *Madurella*. Brumpt has distinguished several varieties of parasite concerned in Madura disease, and finds that a pale variety may be produced by a hyphomycete as well as by Vincent's actinomyces ; in fact, with the exception of Vincent's organism, all the parasites have been considered by him to be closely allied to *Aspergillus*.

Methods of Examination and Diagnosis.—As actinomycosis cannot be diagnosed with certainty apart from the discovery of the parasite, a careful examination of the pus in obscure cases of suppuration should always be undertaken The colonies may be recognised with the naked eye, especially when some of the pus is spread out in a thin layer on glass and held up to the light If they are scanty, their detection is aided by liquefying the pus with caustic soda solution and then examining the sediment obtained by centrifuging If the granules are washed in salt solution and examined unstained, the clubs, if present, are at once seen on microscopic examination. Several examinations, however, may be necessary before granules are found To study the filaments, a colony should be broken down by crushing it between two slides or under a cover-slip on a slide, dried, and stained by Gram's method. While films of pus made at random in the usual way and suitably stained may in certain cases reveal the organism, it is essential that actual granules or colonies should be examined In the case of sections of the tissues, Gram's method with a counterstain yields excellent staining of the mycelium. In the ox the clubs are strikingly demonstrated by staining with carbol-fuchsin and then decolorising with picric-alcohol ; or the preparation may be decolorised with 1 per cent. sulphuric acid and then counterstained with methylene-blue.

Cultures should be made both under aerobic and anaerobic conditions on glucose- or glycerol-agar plates and in shake cultures, also on Löffler's serum. Magnusson recommends keeping the cultures in an atmosphere of CO_2. The medium should be inoculated directly with whole colonies from the pus. Owing to the slow growth of the actinomyces, however, the obtaining of pure cultures is somewhat difficult, unless the pus is free from contamination with other organisms. When other organisms are present the granules should be picked out with a loop and added to sterile saline in a tube, then washed with several changes of saline. They are then shaken in absolute alcohol for half a minute, washed again in three changes of saline, and finally transferred to the culture medium. M. Gordon has recommended for primary cultures from pus, the use of blood

broth (in tubes) under a layer of sterile paraffin to secure anaerobiosis if necessary. In this medium the organism grows well in the form of granules. If a mixed growth results with other organisms present, the colonies in the medium, after washing with sterile saline, are transferred to plates, and in this way a pure growth can be obtained. In infections with Actinobacillus the serum acquires agglutinins for the organism.

Allied Organisms

Leptothrix.—The biological relationships of this type of organism are dealt with in Chapter I. (p. 36). The designation, leptothrix, has been applied to those filamentous organisms which generally resemble the streptothrices, but differ from the latter in the absence of branching. A leptothrix type is a common inhabitant of the mouth and has been spoken of as *L. buccalis*; it may be found in the tartar deposits on the teeth This organism seems to flourish especially in the pockets between the gums and teeth in pyorrhœa alveolaris and its growth has been regarded as a factor in tartar formation which is so marked in pyorrhœa (Bulleid). A leptothrix may also occur in the tonsillar crypts. Concretions in the lachrymal duct have been found to be largely composed of growths of this type of organism. We have observed a "mycosis" of the tongue produced by a leptothrix. Leptothrices have also been recorded in inflammatory and suppurative conditions in the region of the mouth and throat, but the pathogenicity of these organisms seems to be relatively weak. Gifford has described a leptothrix in a case of recurrent conjunctivitis. The leptothrix group has not been studied in sufficient detail from the biological standpoint to merit any classification. The organisms are usually observed in the form of elongated unbranched filaments which are typically Gram-positive. Shorter forms may also be noted similar to large bacilli. They are usually non-motile. Both aerobic and anaerobic strains have been described. Cultures on gelatin may produce liquefaction. Pigment-producing types have been noted. Free spores have also been observed (Gifford). Mackenzie has described in cases of meningitis partially Gram-positive leptothrices, which in certain stages showed motility and which also formed spores. They grew readily on ordinary media, after isolation on blood agar, but had no action on the usual fermentable substances and did not form indole. In some cases the organism was recovered from the blood as well as the cerebro-spinal fluid.

Erysipelothrix.—An organism of this group (*Erysipelothrix* or *Bacillus rhusiopathiæ*) is responsible for a disease of pigs known as "swine erysipelas." Cases of human infection ("erysipeloid") by the organism have been observed, the skin usually being affected. The bacillus of swine erysipelas has also been found in the tonsils, intestine, and fæces of apparently healthy pigs. Though potentially pathogenic, it is apparently a normal inhabitant of the alimentary tract of these animals. In the acute form of the disease there is a general infection, and the organism can be demonstrated in the blood. A characteristic of the disease is the occurrence of multiple patches of congestion, and often hæmorrhages, in the skin—hence the designation "erysipelas." In chronic cases there may be a verrucose endocarditis. In some cases arthritis occurs. The

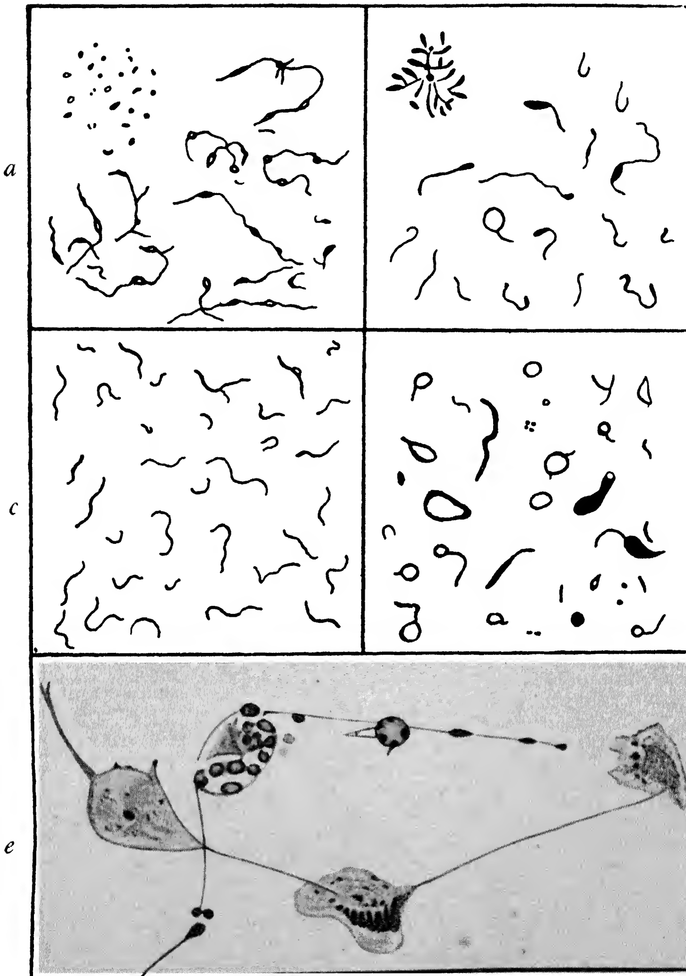


FIG. 97.—Growth Phases of the Organism of Pleuro-pneumonia: *a*, filterable spore-like initial corpuscles and filamentous branched elements (the first vegetative stages); *b*, "actinomycotic" rosette springing from central initial corpuscle, also various filamentous forms (some with nodes); *c*, filamentous forms; *d*, filaments, spheres, rings (produced by curls in filaments) and yeast-like bodies (*a*–*d* from serum-broth cultures); *e*, impression-preparation of young culture on serum-agar, showing vegetation of initial spores to branched filaments and chromatic nodes, also their coalescence and swelling to form basis of young colony.

Giemsa's stain. ×1500.

(Modified from Ledingham, *Journ. Path. Bact.*, 1933, xxxviii. 393.)

organism is a slender non-motile Gram-positive bacillus (1-2 μ by 0·2–0·4 μ), but may develop longer filaments and show true branching. It is a micro-aerophilic organism but grows when exposed to the air; it develops on ordinary media. In stab cultures, below the surface projections develop radially from the line of inoculation and give the whole growth an appearance like a test-tube brush. Colonies on the surface of solid media are very small and delicate, and rarely exceed half a millimetre in diameter. The disease has been reproduced in pigs by inoculation of cultures. Inoculation of mice produces a general infection which is fatal within a few days A similar organism (*E. murisептica*) has been found in a septicæmic disease of mice Treatment by a specific immune serum should be employed in human cases.

The Organisms of Bovine Pleuro-pneumonia and Agalactia of Sheep and Goats.—The organism of bovine pleuro-pneumonia forms a link between the pathogenic lower bacteria and the viruses. It grows readily in an ordinary culture medium such as broth, to which 10 per cent. ox or horse serum has been added, as Nocard and Roux showed, and in such cultures it develops visible structures stainable by the usual methods. On the other hand it exists in forms capable of passing through filters which hold back the ordinary bacteria. Growth is visible after two or three days' incubation at 37° C.; a slight opacity develops in the medium and becomes intensified, until on shaking silky waves of opacity are seen in the fluid. On a similar medium solidified with agar, surface growths may be got in the form of isolated transparent colonies, which after four to five days at 37° C, reach a diameter of about 0·2 mm., with a granular brownish surface and opaque centre. Finally the colony is 1 mm. in size; it is whitish, umbilicate and can be detached from the medium only with difficulty. Acid is produced from a number of fermentable substances, but indole is not formed Cultures die in a few weeks, the development of acid in the medium, *e.g* from the fermentation of glucose, hastening death. In successive subcultures the virulence gradually becomes attenuated. Heating for one hour at 58° C., kills cultures. Borrel with others recognised that this organism showed great pleomorphism and that certain forms appeared in succession in cultures. It is Gram-negative at all stages and requires for its satisfactory demonstration fairly intense staining, *e.g.* with warm Giemsa's solution. The tenacious character of the colonies, due to a mucoid matrix which is formed, has hindered the elucidation of the microscopic appearances (Fig 97). Ledingham has recently used the method of impression-preparations in order to study the growths. Starting from the filterable elements, which are regarded as consisting of condensed but plastic masses of free protoplasm essentially similar to those composing non-filterable elements, filaments grow out which show branching. Then the filaments, either at their ends or in their course, may produce beads of great variety in size and shape and with intense affinity for chromatin stains. From these by budding there arise rings, spheres, vibrios, filaments, and other forms seen in cultures at the height of growth. The relation of the latter forms to each other is not clear, however. Around the deeply staining material which with Giemsa's solution assumes the purple colour of chromatin, a pale blue-stained sheath is seen. Ledingham

considers that the organism shows close affinities with the actinomyces

Agalactia, a contagious disease of sheep and goats which causes inflammation of the udder, eyes, and joints, is due to a similar organism This can be recovered in pure culture from the affected joints and the related lymph glands.

CHAPTER XIV

ANTHRAX BACILLUS

Introductory.—Anthrax is a disease occurring epizootically among the herbivora, especially sheep and oxen, in which animals it has the characters of a rapidly fatal form of septicæmia, an extensive multiplication of the bacilli in the blood being attended by splenic enlargement. The disease is comparatively rare in the human subject and does not occur as a natural infection from man to man, but may be communicated to him directly or indirectly from animals, and it may then appear in one of three forms. In the human subject, the bacilli are much more restricted to the local lesions than is the case in the ox.

Historical Summary.—Historical researches leave little doubt that from the earliest times anthrax has occurred among cattle. For a long time its pathology was not understood, and it went by many names. In 1849 Pollender observed that the blood of anthrax animals contained numerous rod-shaped bodies which he conjectured had some causal connection with the disease. In 1863 Davaine announced that they were bacteria. He stated that they appeared in large numbers in the blood a few hours before death and that unless blood used in inoculation experiments on animals contained them death of the latter did not ensue. Though this conclusion was at first disputed, still by the work of Davaine and others the causal relationship of the bacilli to the disease had been nearly established when the work of Koch appeared in 1876. This not only did much to clear up the whole subject, but formed the starting-point of the science of bacteriology. Koch confirmed Davaine's view that the bodies were bacteria. He observed in the blood of anthrax animals the appearance of division, and from this deduced that multiplication took place in the tissues. He observed them under the microscope dividing outside the body, and noticed spore-formation taking place. He also isolated the bacilli in pure culture outside the body, and, by inoculating animals with them, produced the disease artificially. Koch's observations were, shortly afterwards, confirmed in the main by Pasteur, though controversy arose between them on certain minor points. Moreover, further research showed that the disease could be produced in animals by feeding them with spores, and thus the way in which the disease might spread naturally was explained.

Bacillus Anthracis. — Microscopical Characters and Cultivation. — This micro-organism is readily grown and

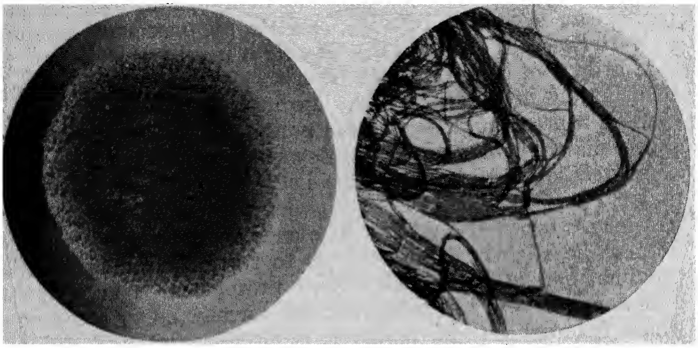
identified, and its characters illustrate the general morphology of the whole group of bacteria. Further, its behaviour when inoculated in animals illustrates in many points the general pathogenic effects of bacteria. Hence an enormous amount of work has been done in investigating it in all its aspects.

If a drop of blood is taken from an auricular vein of an ox immediately after death from anthrax, it will be found to contain a great number of large non-motile bacilli in pairs or short chains. On staining with watery methylene-blue, the characters of the bacilli can be better made out. They are about 1·2 μ thick, and 5 to 8 μ long, though both shorter and longer forms also occur. The ends are sharply cut across, or may be slightly concave so as to resemble somewhat the proximal end of a phalanx. Their protoplasm is very finely granular, and very frequently appears surrounded by a capsule, whose external margin, however, is often not so well defined as in the case of the pneumococcus. When several bacilli lie end to end in a chain, the capsule seems common to the whole chain. They stain well with all the basic aniline dyes and are Gram-positive. The capsule may be demonstrated by the usual methods (p. 122) or it may be stained with aniline-crystal-violet solution,[1] rapidly differentiated in water acidulated with acetic acid, and mounted in water.

Methylene-blue Reaction.—This was introduced independently by McFadyean and by Heim with a view to the easy recognition of the bacilli in blood or other body fluids, and depends on a disintegration of the bacillary capsules which occurs when these are imperfectly fixed. Imperfect fixation is attained by drying a blood film on a *slide* and holding it three times for a second in a flame, film upwards (too great heating fixes the capsules and prevents the reaction from occurring). The preparation is stained for a few seconds with an old solution of methylene-blue, 1 per cent. in water (*i.e.* with a methylene-blue possessing polychrome qualities, see p. 116) It is washed in water and dried with filter-paper—a cover-glass is not applied. In such a preparation, between and near the bacteria there is a varying amount of an irregularly disposed amorphous or finely granular material of a violet or reddish-purple tint. Frequently the colour reaction in the preparation is so marked as to be recognisable by the naked eye. McFadyean states that this reaction does not occur with putrefactive or other bacteria which might be present under circumstances where the recognition of the anthrax bacilli is the question under consideration.

[1] This is prepared by mixing, shortly before use, 1 volume of a saturated alcoholic solution of the dye with 10 volumes of aniline water (the latter is made by adding 5 c.c. aniline to 100 c.c. distilled water in a flask, shaking vigorously, and filtering through a filter-paper previously moistened with water—the solution should be kept in the dark). The watery stain should be filtered.

Plate Cultures.—From a source such as that indicated, it is easy to isolate the bacilli by making agar plate cultures. After



FIGS. 98A, 98B.—Surface colony of the anthrax bacillus on an agar plate—A, under a low power, showing the characteristic appearance (×7). B, impression-preparation of the margin of the colony. Stained by Gram's method. ×300.

twelve hours at 37° C., colonies are recognised under a low power as wavy wreaths, like locks of hair, radiating from the centre and apparently terminating in a point (Fig. 98A). On examining with a high power, the wreaths are seen to be made up of bundles of long filaments lying parallel with one another, each filament consisting of a chain of bacilli lying end to end, and similar to those observed in the blood (Figs. 98B, 99). Graham-Smith (*vide* p. 6) attributes the appearance to the toughness of the bacterial envelope, which prevents the separation of individuals from one another after division. Thus the colony consists of a continuous convoluted chain of bacilli.

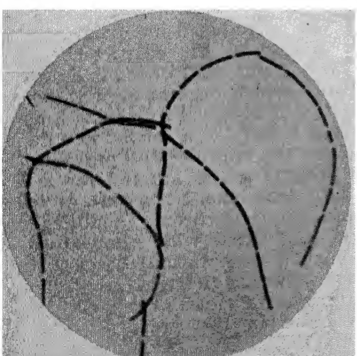


FIG. 99.—Anthrax bacilli arranged in chains, from a twenty-four hours' culture on agar at 37° C.
Stained by Gram's method. ×1000.

The colonies are suitable for making impression preparations which preserve permanently the appearances described. A cover-glass is carefully cleaned and flamed; it is then placed on the

surface of the medium and gently pressed down on the colony. The edge is then raised by a sterile needle, it is held with forceps, dried high over the flame, and treated as an ordinary cover-glass preparation.

On gelatin plates, after from twenty-four to thirty-six hours at 20° C., the appearances are similar; but sometimes, instead of being wreathed at the margin, the colonies give off radiating spikelets irregularly jointed, nodulated, and whorled, which produce a star-like form. These spikelets are composed of spirally twisted threads.

Agar slope cultures exhibit a thick felted growth, the edges of which show a wreathed appearance. By transmitted light the growth presents a ground-glass appearance. The organism grows readily on *potato*, but the cultures show no special characteristics. *Coagulated serum* is slowly liquefied by growth of *B. anthracis*.

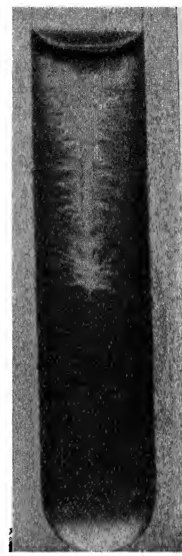


Fig. 100.—Stab culture of the anthrax bacillus in peptone-gelatin; seven days' growth. It shows the "spiking," and also, at the surface, commencing liquefaction. Natural size.

In *gelatin stab* cultures, the characteristic appearance can be best observed when a low proportion, say, 7½ per cent., of gelatin is present, and when the tube is directly inoculated from anthrax blood. In about two days there radiate out into the medium from the needle track numerous very fine spikelets which enable the cultures to be easily recognised. These spikelets are longest at the upper part of the needle track; thus the "inverted fir tree" appearance results (Fig. 100). Spread takes place on the surface of the gelatin, and here liquefaction commences, and gradually extends down the stab and out into the medium, till the whole of the gelatin may be liquefied.

While the above are the typical cultural appearances met with in virulent cultures, *variants* may occur either spontaneously or as the result of growth under special conditions (p. 492).

In *broth* after twenty-four hours' incubation at 37° C., there is usually the appearance of irregular spiral threads suspended in the liquid, which are made up of bundles of parallel chains

of bacilli. Later, growth is more abundant, and forms a flocculent mass at the bottom of the fluid.

B. anthracis ferments glucose, saccharose, and maltose with acid but without gas production.

The Biology of B. Anthracis.—Koch found that *Bacillus anthracis* grows best at a temperature of 35° C. Multiplication does not take place below 12° C. or above 45° C. In the spore-free condition the bacilli have comparatively low powers of resistance. They do not stand long exposure to 60° C., and if kept at ordinary temperature in the dry condition they are usually found to be dead after a few days. The action of the gastric juice is rapidly fatal to them, and they are accordingly destroyed in the stomachs of healthy animals. They are also soon killed in the process of putrefaction. They can, however, be cooled below the freezing-point without dying. The organism is a facultative anaerobe; but grows best in the presence of oxygen, and some of its vital functions are best carried on in the presence of this gas. Thus in gelatin stab cultures the liquefaction always commences at the surface and spreads downwards. Spore formation does not occur in the absence of oxygen.

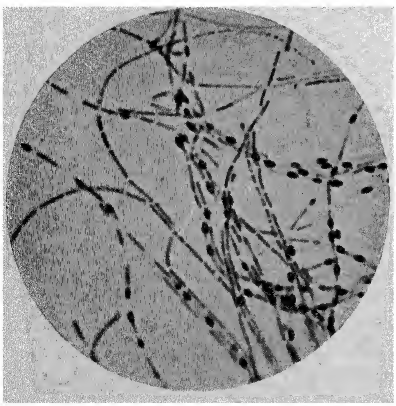


FIG. 101.—Anthrax bacilli containing spores (the darkly coloured bodies); from a three days' culture on agar at 37° C. See also Plate III., Fig. 11.
Spore stain. ×1000.

Sporulation.—Under certain circumstances sporulation occurs in anthrax bacilli. A little highly refractile speck appears in the protoplasm about the centre of the bacillus; this gradually increases in size until it forms an oval body of about the same thickness as the bacillus, lying in the bacillary protoplasm (Fig. 101). The latter gradually loses its staining capacities and finally disappears. The spore thus lies free as an oval highly refractile body which does not stain by ordinary methods, but which can be stained by special methods (p. 122). When the spore is again about to assume the bacillary form, polar

rupture of the capsule occurs, and the contents grow out, taking on the ordinary rod-shaped form.

It is generally agreed that sporulation never occurs within the body of an animal suffering from anthrax. Koch attributed this to the absence of free oxygen, which he found necessary to the occurrence of spores in cultures outside the body. Another factor is necessary to sporulation, namely, a suitable temperature. The optimum temperature for spore production is 30° C. Koch found that spore-formation did not occur below 18° C. Above 42° C. not only does sporulation cease, but Pasteur found that if bacilli were kept at this temperature for a time they did not regain the capacity when again grown at a lower temperature. In order to make them again capable of sporing, it was necessary to adopt special measures, such as passage through the bodies of a series of susceptible animals. Non-sporing variants may also be isolated from sporing strains, especially when those opaque portions of growth are selected which develop late on old agar cultures. Bordet has shown that the production of non-sporing cultures is favoured by growing the organisms on media rich in calcium (*vide infra*).

Anthrax spores have extremely high powers of resistance. Koch found they resisted boiling for five minutes; and dry heat at 140° C. must be applied for several hours to kill them with certainty. In a dry condition at ordinary room temperature and exposed to diffuse daylight 50 per cent. of spores cease to be capable of germinating in a few months, while a considerable proportion of the remainder is still able to germinate after ten years (Graham-Smith). Unlike the bacilli, they can resist the action of the gastric juice for a long period of time. When present on hides they survive lime tanning, but on the addition of 1 per cent. sodium sulphide to the lime bath, provided the temperature is not below 23° C., they are unlikely to resist ten days' treatment (Robertson). They are often used as test objects by which the action of germicides is judged (see Chapter XXIX.). It is to be noted, however, that spores derived from a given culture possess varying resistance among themselves; also the spores formed by different strains of the organism may vary markedly in this respect.

Capsulation.—This is very frequently observed in *B. anthracis* both in tissues and in cultures (Fig. 102), but the appearances vary under different biological conditions and sometimes capsule formation is absent. The capsule sometimes has as sharp an external contour as occurs in the pneumococcus, but in other cases is not so definitely marked, and sometimes when bacilli

are lying together their capsules appear to blend to form a somewhat ill-defined halo. Such variations are associated with slight differences in the naked-eye appearance and physical characters of surface growths. In those where the capsule is indefinite, the growth is moister and more slimy and the edges of the colonies may not present the typical wreathed appearances already described. Such variations have been noted by Preisz as of special frequency in strains deprived of their power of sporulation by heat, and different colonies isolated from such strains may present differences in the character of the capsule. It has been shown that capacity to produce a well-formed firm capsule *in vivo* is associated with the possession of special virulence, non-capsulating strains frequently showing weakly pathogenic qualities. In this connection it is important that the capsulated forms are insusceptible to phagocytosis, while the non-capsulated are readily taken up by phagocytes. The capsule contains a characteristic protein but is free from polysaccharide. On the other hand, polysaccharide can be demonstrated in the bodies of non-capsulated as well as capsulated anthrax bacilli (Tomcsik and Szongott).

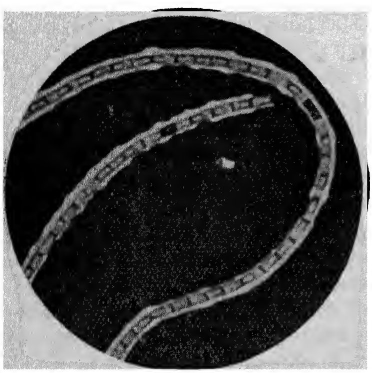


Fig. 102. — Anthrax bacilli showing capsule. Eosin capsule method. ×1000.

Variants.—Highly virulent strains present the typical appearances already described, *i.e.* the colonies are large, "rough," wreathed at the edge, and made up of bacilli in continuous chain formation. Variants present a smaller and smoother type of colony without the typical wreathed margin, the bacilli being arranged in pairs or bundles; they are less virulent than the rough type. Repeated sub-culturing of the rough type on agar containing calcium salts leads to diminution in sporing forms and finally to a filamentous non-sporing strain which is avirulent. The growth of this non-sporing organism on media decalcified by the addition of oxalate leads to the formation of mucoid variants.

It is evident from what has been said that modifications in

both biological and cultural characters can be artificially originated in anthrax bacilli. Spores which are kept in the dry state under ordinary conditions for many months, *e.g.* by letting an agar culture dry up, usually yield virulent cultures. A good method of preserving the virulence and sporing property of *B. anthracis* is to maintain it in cultures on agar containing 0·1 to 0·2 per cent. neutral sodium oxalate (Bordet).

Anthrax in Animals.—Anthrax occurs from time to time epizootically in sheep and cattle, and may also attack goats, horses, deer, and camels; it is world-wide in its distribution. All the countries of Europe are from time to time visited by the disease, but in some it is much more common than in others. In Britain the death-rate is small, and often only one animal in a herd is affected, but in France the annual mortality among sheep was formerly about 10 per cent. of the total number in the country, and among cattle 5 per cent. The incidence, however, has been greatly reduced—a result ascribed to the system of preventive treatment (*vide infra*). In sheep and cattle the disease is specially virulent, and death often occurs with great rapidity. In less acute cases the animal is apparently out of sorts, and does not feed; there is often a sanguineous discharge from the bowels, and bloody mucus may be observed about the mouth and nose. Progressive weakness, with cyanosis, is followed by death in from twelve to forty-eight hours. When the disease is more prolonged, widespread œdema and extensive enlargement of lymphatic glands are marked features; and in the glands, especially about the neck, actual necrosis with ulceration may occur, constituting the so-called anthrax carbuncles. Such subacute conditions are especially found among horses, which are by nature not so susceptible to the disease as cattle and sheep. In affected cows the bacilli may be present in the milk. Occasionally even in susceptible animals recovery takes place.

On post-mortem examination of an ox dead of anthrax, the most noticeable feature—which has given the name "splenic fever" to the disease—is the enlargement of the spleen, which may be two or three times its natural size. It is of dark red colour, and on section the pulp is very soft and friable. A film made from the spleen and stained with watery methylene-blue will be found to contain enormous numbers of bacilli mixed with red corpuscles and leucocytes, chiefly lymphocytes and the large mononucleated variety (Fig. 103). The lymphatic system generally is much affected, especially in less acute cases. The glands, especially the mediastinal, mesenteric, and cervical

glands, are enlarged and surrounded by œdematous tissue, the lymphatic vessels are swollen, and both glands and vessels may contain innumerable bacilli. The intestines are enormously congested, the epithelium more or less desquamated, and the lumen filled with a bloody fluid. The changes in other organs are those met with in septicæmia. The blood throughout the body is usually fluid and of dark colour; bacilli can be found in it on microscopic examination, but they are specially numerous in the capillaries of internal organs.

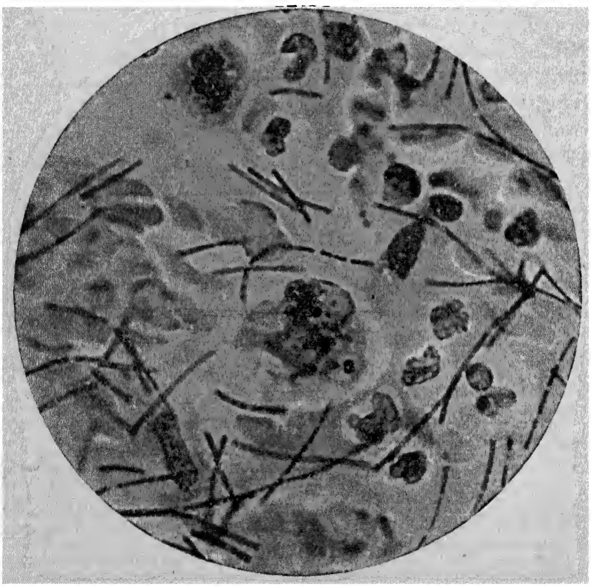


FIG. 103.—Scraping from spleen of guinea-pig dead of anthrax, showing the bacilli mixed with leucocytes, etc. (Same appearance as in the ox.)
Wet fixed film stained with carbol-thionin. ×1000.

Great differences exist in susceptibility to anthrax in different species of animals. Thus the sheep (except those of Algeria, which only succumb to enormous doses of the bacilli), guinea-pig, and mouse are all very susceptible, the rabbit slightly less so. The ox is highly susceptible to the natural infection, but much less so to subcutaneous inoculation. Less susceptible than this group are the horse, deer, and goat, in which the disease occurs from time to time in nature. Anthrax also occurs epizootically in the pig, often from the ingestion of the organs of other animals dead of the disease. A careful bacteriological examination is here always advisable,

especially of any œdematous infiltration about the throat, or in the neighbouring lymphatic glands; often, in pigs dying of anthrax, bacilli may not occur in the blood. Any hæmorrhagic infarction in the spleen of a suspected animal should be carefully investigated. The human subject may be said to occupy a medium position between the highly susceptible and the relatively immune animals. The white rat is much less susceptible to the infection than the brown rat. Adult carnivora are also very resistant, and birds and amphibia are in the same position. In the tissues of birds inoculated with spores, the organisms appear to be rapidly killed.

With these differences in susceptibility there are also great variations in the pathological effects produced in the natural or artificial disease. This is especially the case when we consider the distribution of the bacilli in the bodies of the less susceptible animals. Instead of the widespread occurrence described above, they may be confined to the point where they first gained access to the body and the lymphatic system in relation to it, or may be only very sparsely scattered in organs such as the spleen (which is often not enlarged), the lungs, or kidneys.

Experimental Inoculation.—Of the animals commonly used in laboratory work, guinea-pigs and mice are the most susceptible to anthrax, and are generally used for test inoculations. If a small quantity of anthrax bacilli be injected into the subcutaneous tissue of a guinea-pig, a fatal result follows, usually in two to four days. *Post mortem*, around the site of inoculation the tissues, owing to intense inflammatory œdema, are swollen and gelatinous in appearance, small hæmorrhages are often present, and on microscopic examination numerous bacilli are seen. The internal organs show congestion and cloudy swelling, with sometimes small hæmorrhages, and their capillaries contain enormous numbers of bacilli (Fig. 104), as has already been described in the case of the ox; the spleen also shows a corresponding condition. Highly susceptible animals may be infected by being fed with spores or by being made to inhale the bacilli or their spores, a general infection rapidly occurring by both methods. Besredka brought forward observations to show that the guinea-pig and the rabbit are susceptible only to inoculation of the skin; and he also stated that when the bacilli are injected intravenously or intraperitoneally, care being taken to avoid contamination of the skin, no harmful result may occur. His results have not been confirmed generally (see Gratia). The conclusion may be drawn from this work that the conditions for the occurrence of infection are specially favourable when the organisms are inoculated into the skin.

Anthrax in the Human Subject.—As we have noted, man

occupies a middle position in the scale of susceptibility to anthrax. It is always communicated to him from animals directly or indirectly, and usually is seen among those whose trade leads them to handle the carcases or skins of animals which have died of the disease. It occurs in two principal forms, the main difference between which is due to the site of entrance of the organism into the body. In one, the path of entrance is through cuts or abrasions in the skin, or through the hair follicles. A local condition

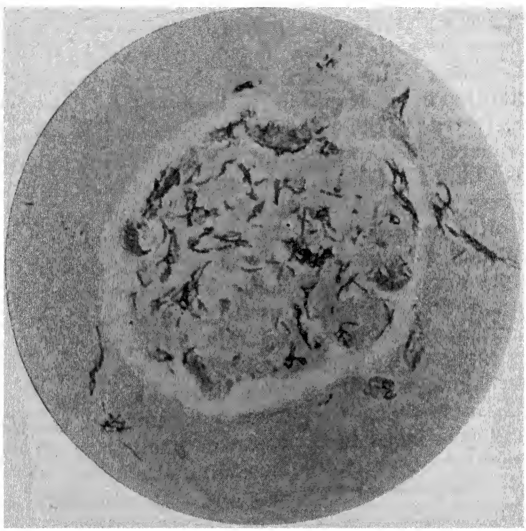


FIG. 104.—Section of kidney of guinea-pig dead of anthrax, showing the bacilli in the capillaries, especially of the glomerulus. Stained by Gram's method and picric acid. ×300.

called a "malignant pustule" develops, which may lead to a general infection. This variety occurs chiefly among butchers and those who work among hides (foreign ones especially). In Britain the workers of the latter class chiefly liable are the hide-porters and hide-workers in South-Eastern London. Occasionally the disease has been contracted from anthrax spores in shaving-brushes made from the bristles of infected animals. In the other variety of the disease the site of infection is the trachea and bronchi, and here a fatal result almost always follows. The cause is the inhalation of dust or threads from wool, hair, or bristles, which have been taken from animals

dead of the disease, and which have been contaminated with blood or secretions containing the bacilli, these having afterwards formed spores. This variety is often referred to as "woolsorter's disease," from its occurring in the centres of the woolstapling trade (in England, chiefly in Yorkshire), but it also is found in places where there are hair, brush, or carpet factories.

(1) *Malignant Pustule.*—This usually occurs on the exposed surfaces—the face, hands, forearms, and back, the last being a common site among hide-porters. One to three days after inoculation a small red painful pimple appears, soon becoming a vesicle, which may contain clear or blood-stained fluid; it is rapidly surrounded by an area of intense congestion. Central necrosis occurs, and leads to the malignant pustule proper, which in its typical form appears as a black eschar of irregular shape often surrounded by a ring of vesicles, these in turn being surrounded by a congested area. From this pustule as a centre subcutaneous œdema spreads, especially in the direction of the lymphatics; the neighbouring glands are enlarged. Sometimes there may be marked local œdema without a recognisable "pustule." There is usually fever with general malaise. On microscopic section of the typical pustule, the central eschar is noticed to be composed of necrosed tissue and degenerating blood cells; the vesicles are formed by the raising of the stratum corneum from the rete Malpighii. The cells of the latter are swollen and œdematous, the papillæ being enlarged and flattened out and infiltrated with inflammatory exudation which also extends beneath the centre of the pustule. In the tissue next the eschar necrosis is commencing. The subcutaneous tissue is also œdematous, and often infiltrated with leucocytes. The bacilli exist in the periphery of the eschar and in the neighbouring lymphatics, and, to a certain extent, in the vesicles (Fig. 105). They may be very scanty being found in sections, although films and cultures from scrapings had proved negative. It is very important to note that widespread œdema of a limb, enlargement of neighbouring glands, and fever may occur while the bacilli are still confined to the immediate neighbourhood of the pustule. Sometimes the pathological process goes no further, the bacilli gradually die out, the eschar becomes a scab, the inflammation subsides, and recovery takes place. In other cases, however, the œdema spreads, invasion of the blood stream may occur, and the patient dies with, in a modified degree, the pathological changes detailed in regard to the acute disease in cattle. In man the spleen

is usually not much enlarged, and the organs generally contain few bacilli.

(2) *Woolsorter's Disease.*—The pathology of this affection was first worked out in this country by Greenfield. The local lesion is usually situated in the lower part of the trachea or in the large bronchi, and is in the form of swollen patches in the mucous membrane, often with hæmorrhage into them—small ulcers may also be seen. The tissues are intensely inflamed,

FIG. 105.—Section of a malignant pustule at the periphery, showing numerous anthrax bacilli. Stained by Gram's method and carmalum. ×300. (From a preparation by Professor J. Shaw Dunn.)

œdematous, and the cellular elements are separated, but there is usually little or no necrosis. There is enormous enlargement and engorgement of the mediastinal and bronchial glands, and hæmorrhagic infiltration of the cellular tissue in the region. There are pleural and pericardial effusions, and hæmorrhagic spots occur beneath the serous membranes. The lungs show great congestion, collapse and œdema. There may be cutaneous œdema over the chest and neck, with enlargement of glands, and the patient rapidly dies with symptoms of pulmonary embarrassment, and with a varying degree of pyrexia. It is to be noted

that in such cases, though numerous bacilli are present in the bronchial lesions, in the lymphatic glands, and affected tissues in the thorax, comparatively few may be present in the various organs, such as the kidney, spleen, etc., and sometimes it may be impossible to find any.

(3) *Intestinal Anthrax.*—Infection occasionally takes place through the intestine, probably by ingestion of spores as in the case of animals; but this condition is rare. In such cases there occur single or multiple local hæmorrhagic lesions in the intestinal mucous membrane, the central parts of the hæmorrhagic areas tending to be necrotic and yellowish, and there may be a corresponding affection of the mesenteric glands.

A considerable number of cases have been recorded in which *hæmorrhagic meningitis*, associated with the presence of the anthrax bacilli in large numbers, has occurred as a complication of various primary lesions.

The Spread of the Disease in Nature.—We have seen that the *B. anthracis* rarely, if ever, forms spores in the body, and if the bacilli could be confined to the blood and tissues of carcases of animals dying of the disease, it is certain that anthrax in an epizootic form would be less frequent. For it has been shown by many observers that in the course of the putrefaction of such a carcase the anthrax bacilli rapidly die out, and that after ten days or a fortnight very few remain. But it must be remembered that while still alive an animal is discharging by the bloody excretions from the mouth, nose, and bowel, myriads of bacilli which may rapidly spore, and thus arrive at a very resistant stage. These lie on the surface of the ground and are washed off by surface water. It is in the condition of spores that they are dangerous to susceptible animals. In the bacillary stage, if swallowed, they will be killed by the acid gastric contents; but as spores they can pass uninjured through the stomach, and gaining an entrance into the intestine, infect its wall, and ultimately reach, and multiply in, the blood. It is known that in the great majority of cases of the disease in sheep and oxen, infection takes place thus from the intestine. Moist, swampy ground in warm countries may remain infected for years. In Britain infection from soil or pasture is relatively infrequent, and the disease occurs mostly in sporadic form. Cases are most frequent in the winter and among cattle, and the evidence points to infection by imported food-stuffs, *e.g.* maize meal, oil-cake, etc. *B. anthracis* has been demonstrated in such materials.

The Disposal of the Carcases of Animals dead of Anthrax.[1]—It is extremely important that anthrax carcases should be disposed of in such a way as to prevent their becoming future sources of infection. If anthrax be suspected as the cause of death, no post-mortem examination should be made, but only a small quantity of blood removed from an auricular vein for bacteriological investigation. If such a carcase be now buried six feet below the surface of the earth and with a layer of quicklime not less than 1 foot thick surrounding it both above and below, little danger of infection will be run. The bacilli, being confined within the body, will not spore, and will die during the process of putrefaction. The danger of sporulation taking place is, of course, much greater when an animal has died of an unknown disease, which, on post-mortem examination, has proved to be anthrax, but similar measures for burial must be here adopted. In some countries anthrax carcases are burned, and this, if practicable, is of course the best means of treating them. The chief source of danger to cattle subsequently, however, proceeds from the infection of fields, yards, and byres with the offal and the discharges from anthrax animals. All material suspected of being infected should be burned along with the straw in which the animals have lain. The stalls or buildings in which the anthrax cases have been housed, etc., must be washed with a 5 per cent. solution of phenol (or an approved substitute). Needless to say, the greatest care must be taken in the case of men who handle the animal or its carcase that they have no wounds on their persons, and that they thoroughly disinfect themselves by washing their hands, etc., in 1 : 1000 solution of corrosive sublimate or in liquor cresol. sap. All clothes soiled with blood, etc., from anthrax animals, should be thoroughly boiled or steamed for half an hour before being washed.

Immunity against Anthrax.—*Active Immunisation.*—Having ascertained that there was ground for believing that in cattle one attack of anthrax protected against a second, Pasteur (in the years 1880–82) elaborated a method by which a mild form of the disease could be given to animals, and which rendered harmless a subsequent inoculation with virulent bacilli. He found that the continued growth of anthrax bacilli at 42° to 43° C. caused them to lose their capacity of producing spores, and also gradually to lose their virulence, so that after twenty-four days they could no longer kill either guinea-pigs, rabbits, or sheep. Such cultures constituted his *premier vaccin*, and protected against the subsequent inoculation with bacilli which had been grown for twelve days at the same temperature, and the attenuation of which had therefore not been carried so far. The latter constituted the *deuxième vaccin*. It was further found that sheep thus twice vaccinated now resisted inoculation with a culture which usually would be fatal. After the

[1] The method of disposal of anthrax carcases and the treatment of infected premises are prescribed in the Anthrax Order of 1928.

efficacy of vaccination in this way had been experimentally established on a large scale, the method was employed as a preventive measure and has since that time been regularly used in France and elsewhere. It is to be noted that the state of active immunity passes off—in a considerable proportion of cases at the end of a year. Koch found also that vaccinated sheep which resisted subcutaneous inoculation might become infected when fed with spores.

In France, during the twelve years 1882–93, 3,296,815 sheep were vaccinated, with a mortality, either after the first or second vaccination, or during the subsequent twelve months, of 0·94 per cent., as contrasted with the ordinary mortality in all the flocks of the districts of 10 per cent. During the same time 438,824 cattle were vaccinated, with a mortality of 0·34 per cent., as contrasted with a probable mortality of 5 per cent. if they had been unprotected.

The Pasteurian method of vaccination against anthrax apparently yielded most successful results when first applied, but has been subject to a good deal of criticism in recent times. The vaccines are difficult to standardise, and attenuation at 42° to 43° C. may not proceed in the regular manner originally observed by Pasteur. If their virulence is too high, vaccinated animals may die from the inoculation and even spread the disease; if too low, an effective immunity does not result. Following on Besredka's work the intracutaneous method of immunising animals with an anthrax vaccine has been extensively used by Velu; successful results have been reported to follow a single injection.

While larger susceptible animals can be immunised by the methods above described, it has been found difficult or often impossible to produce immunity by this method in the guinea-pig and the rabbit, mainly owing to their susceptibility to a fatal infection when injected subcutaneously with Pasteur's vaccines. Besredka has found that immunity can be developed when inoculation by the skin is employed, either intracutaneous injection or rubbing a culture over a shaved area of skin, the vaccines as used by Pasteur being successively applied, followed by virulent cultures. Grierson has shown that rabbits can be safely and effectively immunised by Besredka's cutaneous method. As a result they can tolerate large doses of virulent organisms injected intravenously. It seems likely, however, that these results depend on the lesser susceptibility of the animal to a fatal infection when the vaccine is introduced into the skin as compared with subcutaneous injection. In the case of a very susceptible animal such as the guinea-pig, the method may fail, however. Mixtures of saponin or digitonin with suspensions of virulent anthrax bacilli or spores when injected subcutaneously into rabbits and large animals cause no serious effects and a single dose leads to immunity (Hruška).

Anti-anthrax sera have been prepared from highly immunised animals, and such sera have been found to possess protective and, to a certain extent, curative properties. In their preparation it is necessary to use living cultures of the bacillus; these are usually employed at first attenuated (by Pasteur's method) and later in the form of virulent cultures. The method has been combined with the use of an antiserum obtained from an animal already immunised—that is, a combination of active and passive immunisation is carried out. The two best known sera are those of Sclavo and Sobernheim. The former, who found the ass the most suitable animal for immunisation, published favourable results obtained in the treatment of malignant pustule. Out of 164 cases there were only ten deaths, this representing about a fourth of the ordinary mortality in Italy. The serum is administered by intramuscular or by intravenous injection. Sabolotnyi has reported favourable results in very severe human cases with bacilli present in the blood, who received the serum intravenously. Similarly, Lucchesi and Gold have treated successfully twenty-eight out of twenty-nine cases, including one in which there were lung lesions and the bacilli were present in the blood. A large intravenous dose of antiserum was given (100–250 c.c.) repeated if necessary at intervals of twenty-four hours, one to six injections being administered in all. According to Kraus and Beltrami normal ox serum also has effect. An important additional point in treatment is to avoid any interference with the local lesion; the part should be kept completely at rest.

Combined immunisation.—Sobernheim's serum has been used for protective purposes in animals, the antiserum being injected at the same time as Pasteur's second vaccine but at a separate site. This method has been widely used in Europe and South America, and it is claimed that it has advantages over Pasteur's, especially in that its application is simpler, one operation instead of two being sufficient.

Mechanism of Immunity to Anthrax.—While anti-anthrax sera have markedly preventive properties, it does not seem possible to explain their action by antibodies which can be demonstrated *in vitro*. Sobernheim and others were unable to detect in such sera special bactericidal properties, and Sclavo came to the conclusion that in the action of the serum substances of the nature of immune-body and complement are not concerned. Opsonic action has been put forward in explanation, but this has not been accepted as sufficient, and the mode of action has not been satisfactorily explained. Anti-anthrax

serum may contain a precipitin for the products of the anthrax bacillus (*vide infra*), but this, of course, does not explain the beneficial effect of the serum. It is a striking fact that from the blood and tissues of immunised animals which have tolerated inoculation with the organisms, living virulent anthrax bacilli can be recovered even after a week.

In connection with the subject of immunity it is to be recognised also that the manner in which the anthrax bacillus produces its pathogenic effects is very imperfectly understood. Toxic action is undoubtedly concerned, as is indicated by the inflammatory œdema which occurs apart from the actual presence of the bacilli, but it has not been found possible to separate toxins from cultures. Filtered broth cultures are almost non-toxic, and the dead bacilli themselves likewise have little effect. The facts accordingly suggest that when invading the tissues the bacilli form toxins such as are not produced in cultures. These toxins were considered by Bail to be of the nature of aggressins (p. 192), and in support of this he stated that the protective action of an anthrax immune serum is due to its containing anti-aggressins. The supposed aggressins have been obtained by centrifuging œdema fluid obtained from infected animals, and then killing any remaining bacilli. Rabbits may be rendered immune by injections of such products, and their serum then protects other animals.

Antibody Reactions : Ascoli's Thermo-precipitin Reaction, etc.— Ascoli's reaction depends on the observation that certain anthrax immune sera produce a precipitin reaction with the products of the *B. anthracis*. The suspected blood or tissue is boiled for a few minutes in 5 to 10 volumes of normal saline which may be acidulated by one part per thousand of acetic acid ; the fluid is cooled and filtered through paper or asbestos so as to obtain a clear filtrate ; a little of this is then run on to the top of the serum, and a white ring should form immediately at the junction of the fluids. The reaction sometimes occurs with normal sera, but in this case does not appear for a quarter of an hour. It is absolutely necessary that the serum to be used should be previously tested with material derived from an undoubted anthrax case, as only a certain small proportion of immune sera will give the reaction. The reaction seems to depend on an effect produced between the serum and substances derived from the bacilli, as it is most marked with tissues containing numerous organisms. It can be obtained with material which has been kept for six months, and numerous controls made with tissues of animals dying from other diseases are stated to have given negative results. A method of obtaining active antisera for the precipitin test has been described by Rosenberg and Romanow.

Tomcsik and others have shown recently that the capsular protein and the somatic polysaccharide of the anthrax bacillus possess hapten properties, each of which is distinct, although the

same polysaccharide occurs also in other members of the group Non-capsulated bacilli develop antisera which react only with the polysaccharide. Antisera developed by injecting the capsulated bacilli, after being absorbed with a non-capsulated culture, act as a pure antibody to the capsular protein. The latter antibody gives precipitation with high dilutions of the soluble protein and also the complement-fixation reaction ; it produces changes in the capsules which can be detected by the microscope, and agglutinates the capsulated bacilli. By the use of capsular antibody it has been shown that the capsular material formed by the bacilli in the tissues after inoculation with a virulent non-capsulated culture, is serologically identical with that formed in cultures by strains which produce a capsule when growing on agar.

Methods of Examination.—(*a*) *Microscopic Examination.* — In a case of suspected malignant pustule, film preparations should be made from the fluid in the vesicles or from a smear from the surface and stained by Gram's and McFadyean's methods. In this way practically conclusive evidence may be obtained. Occasionally bacilli are so scanty that both film preparations made from different parts and even cultures may give negative results. Care ought to be taken in manipulating a pustule, as, otherwise, the diffusion of the bacilli into the surrounding tissues may be aided. The examination of the blood in cases of anthrax in man usually gives negative results, with the exception of very severe cases, when a few bacilli may be found in the blood shortly before death.

(*b*) *Cultivation.*—The material should be stroked on agar plates After twenty-four hours at 37° C. colonies of *B. anthracis* will appear, and from their wavy margins can be readily recognised by means of a hand-lens.

While the isolation of the *B. anthracis* from fresh material is usually easy, great difficulty may be encountered where the organism is to be sought for in an animal which has been dead for twenty-four hours or longer, as the bacilli rapidly die out or are associated with putrefactive organisms. Where cultures cannot be made at once from blood or tissue fluids, the material should be preserved by smearing it on unglazed earthenware and allowing it to dry.

(*c*) *Test Inoculation.*—In the case of hair or hides the suspected material is soaked in sterile water and then heated at 75°–80° C. for fifteen minutes, during which time the material is well teased out or scraped. After the mixture has stood for about an hour, it is centrifuged and the sediment injected subcutaneously into one or two guinea-pigs (mice are less satisfactory). If anthrax bacilli are present, the animals usually die within four days, with the changes in internal organs already described, and the bacilli can be demonstrated in the heart blood. The identification of an organism as the anthrax bacillus cannot be said to be substantiated till its pathogenicity has been proved. When death occurs from a mixed infection with anaerobes, the anthrax bacillus may be recovered *post mortem* by making plate cultures from the peritoneal fluid. Another method is to inoculate contaminated material into the scarified skin, as the anthrax bacillus is more likely to cause infection by this route than are the other pathogens which may be present.

Organisms Biologically allied to the Anthrax Bacillus

It must be recognised that *B. anthracis* constitutes one species among a group of Gram-positive, aerobic, sporing bacilli which are widely distributed in nature. The anthrax bacillus is the only actively pathogenic representative of the group. The others are saprophytes, occurring in soil, dust, water, etc. Spores of these organisms are practically ubiquitous and frequently contaminate culture medium, especially when exposed to air. Some of them so closely resemble *B. anthracis* that, apart from animal inoculation tests, they may be easily mistaken for it. Thus morphological and cultural characters almost identical with those of the anthrax bacillus may be presented by certain of these organisms (*e g.* the so-called *B. anthracoides*) ; though in some cases motility in culture clearly distinguishes them from *B. anthracis*. Inoculation of a mouse or guinea-pig, to which *B. anthracis* is extremely virulent, serves as the essential method of differentiation. It is noteworthy, however, that certain organisms of this type (*e.g. B. anthracoides*) are not entirely devoid of virulence, and may on subcutaneous inoculation produce a lethal effect in mice and guinea-pigs, if large doses of cultures are used (Grierson). Even a septicæmic condition may result, as in anthrax, but the organisms in the blood and spleen fail to show McFadyean's reaction. A small dose of a recently isolated anthrax bacillus is, of course, sufficient to cause death in a susceptible animal.

Classical types belonging to this group are *B. mycoides*, *B. subtilis*, *B. mesentericus* (*vulgatus*). *B. mycoides* is very similar in morphology to *B. anthracis*, but its colonies on nutrient agar differ from those of the latter in exhibiting a feathery or spiked appearance. *B. subtilis* also resembles the anthrax bacillus in morphology, but the ends are often rounded, there is less tendency to chain formation, and in young cultures the bacilli show active motility due to peritrichous flagella. The spores may be central or subterminal. This organism is strictly aerobic and grows well at low temperatures, though the optimum is 37° C. A stroke inoculation on culture medium produces an abundant dry, opaque, wrinkled, greyish-white layer of growth without the characteristic wavy or wreathed margin of *B. anthracis* cultures. A potato culture consists at first of a moist layer of growth, but later assumes a dry mealy appearance. According to Axenfeld, *B. subtilis* may produce infection of the eye with resulting iridocyclitis and panophthalmitis. *B. mesentericus* closely resembles *B. subtilis*, and is characterised by the marked wrinkling and folding of the growths on artificial media. Another member of this group is *B. megatherium*.

CHAPTER XV

THE COLI-TYPHOID GROUP AND RELATED ORGANISMS

Introductory.—The organism known as the *Bacillus typhosus* was first described in 1880–81 by Eberth, who observed its microscopic appearance in the intestinal ulcers and in the spleen in cases of typhoid fever. It was first isolated (from the spleen) in 1884 by Gaffky, and its cultural characters were then investigated. In 1885 Escherich described the *Bacillus coli communis*, the classical type of those organisms designated collectively *B. coli*, which occur normally in the intestine and to which the typhoid bacillus is biologically related. While ordinarily *B. coli* is a harmless commensal, it may manifest pathogenic properties under certain conditions. These two bacilli belong to a large group of organisms (often designated the coli-typhoid group) isolated from various intestinal infections, which bear resemblances to one another, and whose differentiation is in certain cases a matter of some difficulty. Among other members of this group are the paratyphoid bacilli, *B. enteritidis* of Gaertner and other organisms associated with a form of " food poisoning," and the dysentery bacilli.

The general characters of the *coli-typhoid* group are as follows : the organisms, which are microscopically indistinguishable except in regard to motility (and the possession of flagella), this character varying with different members, are small non-sporing bacilli, but in cultures often show variation in length ; their flagella when present are distributed all round the bacillus *i.e.* peritrichous ; they stain with ordinary dyes, and are all Gram-negative ; they are aerobes and facultative anaerobes ; their optimum temperature for multiplication is about 37° C. ; in growth characters on ordinary media they tend to resemble one another, and generally they do not liquefy gelatin, although certain types allied to *B. coli* have the property of gelatin liquefaction (*vide infra*) ; they show wide differences in their actions on sugars, and a primary classification of the group has

been based on the fact that while the typical *B. coli* produces acid and gas from lactose, the typical pathogenic members have no effect on this sugar; in the ultimate differentiation of these organisms various other biochemical properties and *in vitro* immunity reactions are of essential importance.

In this chapter the important organisms to be dealt with will be grouped as follows: (1) those referable to the *B. coli* group, occurring mainly as commensals in the bowel of mammalian animals, including the so-called "*Aerobacter*" types; (2) *B. typhosus* and *B. paratyphosus*, organisms which are associated with enteric fever; (3) the organisms of "food-poisoning" (acute gastro-enteritis) and (4) the dysentery bacilli. It must be recognised, however, that there is no sharp dividing line as regards biological characters between the enteric fever organisms and those of "food poisoning," and the separation of these subgroups is based on their relationships to different clinical conditions.

Bacillus Coli (*Escherichia*)

The designation *Bacillus coli* is used as a collective name applicable to a variety of types presenting well-marked common characters though differing in biochemical reactions and in certain other features. The *B. coli communis* (Escherich) referred to above represents only one type. It is convenient to describe the group as a whole, indicating the differences among the more important types.

Morphological Characters.—These are best seen in young broth or agar cultures. The bacillus is usually 2 to 4 μ long and about 0·5 μ broad; longer forms up to 8 or 10 μ are not infrequent, and short cocco-bacillary forms are also noted. The size may depend greatly on the medium in which the organisms are growing (Figs. 106, 107). Motility varies with different types and under different growth conditions in the same strain. Some strains are capsulate. The organism may stain somewhat faintly with watery dyes, but is readily demonstrated with dilute carbol-fuchsin (1 : 10); it is Gram-negative. In older cultures the bacillary protoplasm may be vacuolated and the organism may appear swollen. By appropriate staining the motile varieties can be shown to possess flagella distributed all round the organism, varying in number, but generally less numerous, shorter, and less wavy than those of *B. typhosus*.

Cultural Characters on Ordinary Media.—A stroke inoculation on *agar* yields after twenty-four hours at 37° C. a

somewhat dense, glistening, white growth along the line of inoculation. In *stab culture in gelatin* an abundant film-like growth takes place on the surface, and there is a whitish line along the stab without liquefaction of the gelatin (Fig. 108). Certain atypical members of the *B. coli* group (*e.g.* *B. cloacæ*) may liquefy gelatin slowly. In *broth*, typical strains produce a uniform turbidity. On *agar plates* the surface colonies are relatively large; the deep colonies are smaller and lenticular in shape and under a low power of the microscope appear rather dense to transmitted light.

FIG. 106.—*Bacillus coli.* Film preparation from a twenty-four hours' culture in glucose broth.
Stained with dilute carbol-fuchsin. × 1000.

Colonies vary in size, thickness, opacity, and structural features among different strains, and in certain cases a mucoid or viscid character associated with the formation of capsules is observed. In fact, certain types (*e.g.* *B. lactis aerogenes*) are characterised by their large, slimy, or mucoid colonies (*vide infra*). "Rough" colonies corresponding to the rough variants noted in other members of the coli-typhoid group may also be observed in some strains (p. 520). On *potato*, in forty-eight hours there is a distinct film of growth, usually of a brownish-yellow tint, with a moist

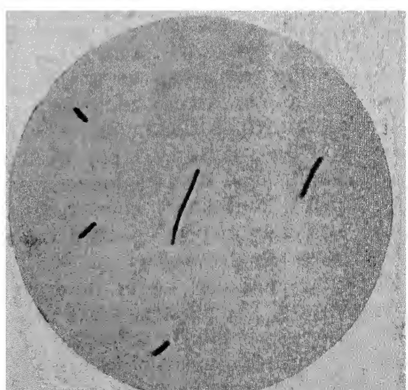


FIG. 107.—*Bacillus coli.* Film preparation from a twenty-four hours' culture in peptone water—the same strain as in Fig. 106.
Stained with dilute carbol-fuchsin. ×1000.

surface, which rapidly spreads and becomes thicker. The appearance on potato, however, varies much with the different strains and also with the reaction of the potato. The optimum temperature for growth is about 37° C. and the temperature range usually from 15° to 45° C., though some types have a lower maximum, *e.g.* 42° C.

Cultural Reactions in Special Media.—Various media have been used for the appreciation of special characters in the *B. coli* group. These reactions depend upon the capacity of the organism to originate chemical changes in a variety of substances.

Fermentative Reactions.—*B. coli* shows great powers of splitting up carbohydrates with the formation of acids, especially lactic acid, and gases, chiefly carbon dioxide and hydrogen. Certain types, however, classified as *B. coli anaerogenes* (*vide infra*) ferment carbohydrates without gas production. As stated, p. 78, peptone water or casein digest with an indicator, *e.g.* litmus, neutral red or Andrade's indicator, in Durham's tubes, is used, the sugar to be employed being added in the proportion of ½ to 1 per cent. The fermentative capacities of *B. coli* are very wide and varied. The common types produce acid and gas from glucose, lactose, lævulose, galactose, maltose, rhamnose, arabinose, mannitol, xylose, glycerol. Certain varieties ferment saccharose, raffinose, dulcitol, adonitol, inulin, cellobiose, dextrin, and even the benzene derivative inosite (see table of sugar reactions, p. 556). Certain glucosides may also be fermented, *e.g.* salicin, arbutin. Some forms of *B. coli* fail to produce gas when growing in MacConkey's fluid medium (p. 891) at 44° C. (Eijkman's test), though typical strains have this property.

FIG. 108.—Stab culture of *Bacillus coli* in gelatin, nine days' growth; the gelatin is split in the lower part owing to the formation of gas.

In cultures in gelatin made from fresh meat sometimes bubbles of gas appear from the fermentation of the dextrose present in the meat (Fig. 108).

The reactions of *B. coli* in some media other than simple sugar solutions likewise depend on sugar fermentation.

Clotting of Milk.—If *B. coli* be grown in milk, *e.g.* litmus

milk, acid is produced from the lactose present, which further curdles the milk. The acid reaction appears to be permanent when growth is allowed to go on for some days.

Measuring of Gas Formation.—As has been said, the gases produced by *B. coli* in fermenting sugars are chiefly carbon dioxide and hydrogen. Many observers have attached considerable importance, first, to the amount of gas formed from a given quantity of glucose in a given time, and, second, to the ratios of the two gases to one another, in such a fermentation. For the observation of this, MacConkey recommended the following method : fermentation tubes (p. 80, Fig. 9), with the closed limb graduated, containing 2 per cent. peptone and 1 per cent. glucose in tap water, are inoculated and incubated for forty-eight hours at 37° C. The tube is allowed to cool and the total amount of gas noted. The bulb is then filled with 2 per cent. sodium hydroxide solution, the opening closed with the thumb and thoroughly shaken. After the gas has been collected in the closed arm the thumb is removed and the ratio of the hydrogen left to the original gas volume is read off. According to Harden and others, coliform bacilli may be classified according to their $CO_2 : H_2$ ratio. In some cases the ratio is approximately 1 : 1–2—the typical coliform bacilli (*vide infra*) ; in others 2–3 : 1, as exemplified by *B. lactis aerogenes* (*vide infra*).

Voges and Proskauer's Reaction.—This is a reaction which is not given by the common types of *B. coli*, but as it occurs with certain members of the coli group (see table) it may be described here. It also depends on carbohydrate fermentation and is due to the formation of acetyl-methyl-carbinol from glucose. A glucose peptone solution tube is inoculated and incubated for two or three days. Then 1 c.c. of a 10 per cent. solution of caustic potash is added and the tube allowed to stand for several hours or overnight at room temperature. A red fluorescent colour is produced, causing the medium to resemble a weak solution of eosin. Acetyl-methyl-carbinol in the presence of free oxygen and alkali undergoes oxidation to diacetyl. The characteristic colour is attributed to a reaction between this product and some constituent of the peptone.

O'Meara's modification of the test is stated to be more delicate than the original method : a minute amount of creatin is added to the culture and then 5 c.c. of 40 per cent. sodium hydroxide, and the tube is shaken. A positive reaction is denoted by the appearance within a few minutes of a pink colour, without fluorescence.

Methyl-Red Reaction.—This depends on the limiting hydrogen-ion concentration attained by growth in a standard glucose peptone medium containing a standard amount of di-potassium phosphate, and has been utilised, along with the Voges-Proskauer reaction, in classifying coliform bacilli isolated from water supplies. The common types found in excreta and sewage produce a high concentration, along with a negative Voges-Proskauer reaction. Methyl-red added to the culture is used as an indicator of the

resulting hydrogen-ion concentration Organisms producing high and low hydrogen-ion concentrations are described as "Methyl-Red Positive" and "Methyl-Red Negative" respectively. For demonstrating the reaction, the organism is grown for two or three days in a peptone water medium containing 0·5 per cent peptone, 0·5 per cent glucose, 0·5 per cent dipotassium hydrogen phosphate; 5 drops of a methyl-red solution (0·1 gram in 300 c.c. alcohol made up to 500 c.c. with distilled water) are added to the culture and the resulting colour noted: a red colour indicates a "positive," a yellow colour a "negative" reaction.

Production of Indole.—The typical forms of *B. coli* produce indole in peptone water. The methods have been given on page 81, and for the detection of the reaction the use of Ehrlich's rosindole test is most satisfactory. Two peptone tubes may be inoculated, and if the reaction is not obtainable in one after two or three days' growth, the other should be incubated for seven to ten days and then tested. Where a faint reaction is obtained, it is well to corroborate the presence of indole by dissolving the rosindole out with amyl alcohol as described.

Citrate-utilisation.—The typical varieties of *B. coli* are unable to use the salts of organic acids as a source of carbon but atypical forms, *e.g. B. lactis aerogenes*, possess this property and are able to grow in a synthetic medium containing sodium citrate. For testing purposes Koser's medium is used: 1·5 grams sodium ammonium hydrogen phosphate, 1 gram potassium dihydrogen phosphate, 0·2 grams magnesium sulphate and 2 grams sodium citrate, made up in a litre of distilled water. Growth in this medium with visible turbidity within ten days indicates utilisation of the citrate.

Action on Neutral Red.—When *B. coli* is grown in neutral-red lactose broth, a rose-red colour, the effect of the acid upon the dye, is at first seen. Frequently this is succeeded by the appearance of a green fluorescence due to a direct action of the organism upon the dye of the nature of a reduction effect. Thus the neutralisation of the acid by an alkali does not lead to a reproduction of the original tint in the indicator. The degree of change, however, varies, the important factors being the percentage of sugar, the reaction, and the strain of the bacillus used. This test is of little value in differentiating types of *B. coli* though at one time used for the purpose.

Reduction of Nitrates.—*B. coli* is generally capable of reducing nitrates to nitrites. For this test, Savage recommended the use of a medium made by dissolving 10 grams of peptone in 1 litre of ammonia-free distilled water, and adding 2 grams of nitrite-free potassium nitrate. The medium is filtered, tubed, and sterilised for half an hour on three days. Tubes are inoculated and incubated for forty-eight hours, the formation of nitrites being now tested for by Ilosvay's method. The following solutions are required: (*a*) sulphanilic acid, 0·5 gram dissolved in 150 c.c. dilute acetic acid (s.g. 1·04); (*b*) 1 gram *a*-naphthylamine is dissolved in 22 c.c of water, the solution filtered, and 180 c.c. dilute acetic acid added In using the test, 2 c.c. of each of these solutions is added to 10 c.c.

of culture. If reduction of the nitrates has occurred, a rose-pink colour should develop almost immediately. It is to be noted that the pink colour first produced sometimes disappears as it is formed or on shaking; in such a case further portions of the two reagents in equal quantities should be added. This test is of little value in the study of *B. coli* types.

Hæmolysis.—According to Dudgeon and others, certain strains of *B. coli* possess distinct hæmolytic properties towards human blood, and these are specially prevalent in acute infections of the urinary system, whereas such hæmolytic strains are uncommon in the fæces.

Agglutination Reactions of B. coli.—A study of the agglutination reactions of this group with the sera of animals immunised with various strains, has generally shown an extreme specificity of the agglutinin, the action of which may be restricted to the individual strain used for immunisation, though it has been claimed that hæmolytic strains derived from urinary infection are serologically related.

Isolation of the B. Coli.—In the case of abscesses or infection of the kidney or bladder, etc. (p. 515), the isolation of the organism is easily accomplished by inoculating plates of MacConkey's bile-salt lactose neutral-red agar (p. 73) with the pus or centrifuged deposit from urine. The colonies are usually characterised by their rose-pink colour. When the organism is present along with other bacteria, as in the case of water, sewage, etc., a bile-salt medium is specially recommended, as it tends to inhibit the growth of organisms except those belonging to the coli-typhoid group. Where there is a mixed infection with such organisms as staphylococci or streptococci, for diagnostic purposes cultures should be made also on other media *e.g.* blood-agar, to obtain a satisfactory growth of these as well as *B. coli*.

The Recognition of Typical B. Coli and Grouping of Coliform Bacilli.—The work on *B. coli*, especially in relation to its occurrence in water, has revealed the existence of a very large number of varieties of the organism. These differ from one another in certain biological characters, *e.g.* motility, fermentation reactions with sugars, indole formation, etc. (*vide infra*). Some difference of opinion has existed as to what characters are to be looked upon as type characters, *i.e.* characters shared by the greatest number of varieties present in fæces.

Two of the original standards may be alluded to. First, that of an English Committee who reported in 1904 on the standardisation of methods for the bacteriological examination of water. According to them, *B. coli* is a small, motile, non-sporing bacillus, capable of growing at 37° C., Gram-negative, never liquefying gelatin, producing clot and permanent acidity of milk within seven days at 37°, fermenting glucose and lactose, with, in both, acid and gas formation—

subsidiary characters being the formation of indole, the formation of a thick yellowish-brown growth on potato, production of fluorescence in neutral-red media, and reduction of nitrates. A similar American Committee looked upon the typical organism as a non-sporing bacillus, motile, fermenting dextrose-broth, with the formation, in the closed limb of the fermentation tube, of about 50 per cent. of gas, of which about one-third is carbon dioxide, causing acid and clot in milk in forty-eight hours, not liquefying gelatin, producing indole, and reducing nitrates.

It may now be said that, in addition to these characters, the common types of lactose-fermenters from the human and animal intestine have no effect on adonitol and inosite, fail to give the Voges and Proskauer reaction, yield a positive methyl-red reaction, and fail to grow in Koser's synthetic medium owing to their inability to utilise citrate.

Varieties of B. Coli

From work done not only with strains isolated from pathological conditions, but in connection with the bacteriology of water, milk, and fæces, it had been found that a very large number of Gram-negative bacilli exist which have the capacity of fermenting glucose and lactose, but which, when further investigated, present individual differences. Much has been done in attempting to differentiate these so-called "lactose-fermenters" from one another. The work of MacConkey first opened the way to such further classification. MacConkey showed that certain of the tests originally applied to the lactose-fermenters in reality gave little significant information, *e.g.* observation of fluorescence in neutral-red lactose media (on account of the inconstancy of the occurrence of this change); the reduction of nitrates—this being a common property of nearly all the members of the group; observation of differences in the appearances of growths—these being very inconstant, and different colonies of the same organisms showing different appearances. On the other hand, important information was obtained by the observation of the indole and Voges and Proskauer reaction (*v. supra*). With regard to sugars MacConkey claimed that in the differentiation of the lactose-fermenters, the only sugars necessary were saccharose, dulcitol, adonitol, inulin, and inosite. By means of these, a preliminary classification could be made from the actions on saccharose and dulcitol.

In his later work MacConkey classified the lactose-fermenters in further detail according to the following criteria: motility, fermentation of dulcitol, saccharose, adonitol, inulin, inosite, the formation of indole, the Voges and Proskauer reaction, and liquefaction of gelatin. This classification elicited the fact that certain biological types were specially prevalent in the fæces of man and animals: *e.g.* the type designated by him No. 71, which corresponds to the organism now spoken of frequently in bacteriological literature as *B. coli communior* (*Escherichia communior*) and *B. coli communis Escherich* (*Escherichia coli*). (See table of characters, p. 556.)

Studies of this group have shown that the most prevalent and characteristic coliform types found in fæces present the following common positive and negative characters: fermentation of lactose with gas production, production of indole, absence of fermentation

of adonitol and inosite, absence of gelatin liquefaction, a CO_2 : H_2 ratio of 1 : 1–2, a negative Voges-Proskauer reaction, a positive methyl-red reaction, and absence of citrate utilisation ; the recognition of these characters becomes of great practical importance in the examination of water for "typical" *B. coli* where these organisms are taken as an index of recent fæcal pollution (p. 892).

Certain atypical varieties of coliform bacilli have in common the property of fermenting inosite, and this affords an important classificatory criterion Such organisms are exemplified by *B. lactis aerogenes* (see Table, p. 556). This type was originally described by Escherich in his work on the intestinal flora of children: he pointed out that it produced gas from milk in the absence of air The separate grouping of the inosite-fermenters is supported by the fact that these organisms usually show other common characters—non-motility, a mucoid capsule, large slimy viscid colonies, fermentation of lactose and adonitol. In addition, the Voges-Proskauer reaction is frequently positive, the CO_2 : H_2 ratio is approximately 2 : 1, the methyl red reaction is usually negative and citrate utilisation is a characteristic The separate designation, *B aerogenes*, is often applied to such organisms. (To this subgroup the capsulate Gram-negative bacilli found in the respiratory tract are closely related, *e.g.* the pneumobacillus, *vide* p. 348)

The question arises as to whether gelatin-liquefying, Gram-negative bacilli which correspond in other characters to *B. coli* are to be included in the *B. coli* group. Among the lactose-fermenters classified by MacConkey certain gelatin liquefiers were represented, e.g. *B cloacæ*, etc., and various authorities have accepted this reaction as one of the possible characters of the group. Such organisms may be regarded, however, as quite atypical (see Table, p. 556).

Thus, among the coliform bacilli there are on the one hand the typical forms, as defined above, which predominate in the fæces of man and animals, and on the other, completely atypical organisms, exemplified by *B. aerogenes*, which are relatively scanty in the intestine of the healthy animal. Between these extremes, however, there are many intermediate atypical forms, which are also relatively infrequent in fæces. From bacteriological studies of water supplies it has been shown that while the typical forms have only a limited power of survival in ordinary environments outside the body, the atypical organisms are often more "resistant" and the latter are therefore found frequently in water, milk, soil, etc., even in the absence of typical *B. coli*. Thus, typical and atypical forms have often been distinguished as "fæcal" and "non-fæcal" respectively, but such nomenclature is somewhat misleading. It must be emphasised that practically all varieties have been found in the intestine ; and atypical forms even when not detectable in fæces by ordinary methods can be demonstrated readily by selective enrichment. Further, in abnormal conditions of the intestine the atypical forms may predominate. The separation of typical from atypical coliform bacilli is nevertheless of the greatest value when *B. coli* is utilised as an indicator of *recent* fæcal contamination. In this case the presence of typical organisms is significant ; the occurrence of atypical forms is of less significance. While the characterisation of the typical *B. coli* given above is generally applicable, the importance attached to particular properties has

varied. Recently special stress has been laid on citrate utilisation as a feature of the non-fæcal types. This property is certainly uncommon among strains isolated from fæces, while it is an almost constant feature of the *B. aerogenes* subgroup. On the other hand citrate-utilising organisms, otherwise typical, may sometimes occur in the intestine; it has been suggested that such organisms should be grouped separately, but this is hardly justifiable. Malcolm has shown from a study of coliform bacilli of bovine origin that all inosite-fermenting strains utilise citrate, while among the non-inosite fermenters there is a close negative correlation between citrate utilisation and the indole reaction. Indole formation has been generally accepted as an important criterion in the recognition of typical *B. coli*. In characterising excretal coliform bacilli from water Bardsley has considered that a negative indole reaction is insufficient by itself to exclude an organism from this category. Another feature of the typical *B. coli* which has been emphasised by some workers is the absence of fermentation of cellobiose, a property possessed by various atypical organisms, but it is doubtful whether this reaction differentiates strictly between "fæcal" and other strains. Recently Wilson and his co-workers have classified strains into groups on the basis of the methyl red, Voges-Proskauer, citrate, indole, Eijkman (*vide* p. 509) and gelatin-liquefaction tests; for details their monograph should be consulted. A further category of rare atypical coliform bacilli is that in which gas production is absent from all carbohydrate fermentations—*B. coli anaerogenes* (see Table, p. 556); the clear differentiation of certain of these from the group of dysentery bacilli (p. 552) presents some difficulty.

Variation and Mutation in the *B. coli* group has been referred to in Chapter I. Variation in fermentative properties is well illustrated by *B. coli mutabilis* in which colonies develop papillæ differing from the parent strain in the power of fermenting lactose, and from these papillæ a stable variant is derived. By an analogous variation a fermentative property may be lost. In certain variable strains different properties may be possessed by different colonies when these are cultivated separately. Variability in respect of practically all characters of these organisms has been recorded and is certainly an outstanding feature of the group. Zinsser and Bayne-Jones have stressed this variability, but while some strains exhibit it, others may remain stable for long periods in culture. It is of course possible that variation is most active in their natural habitat and that laboratory cultures becomes stabilised. Variation in colony characters is also frequently noted, and, as in other groups, there may be dissociation with partial or complete loss of the original antigenic characters.

Pathogenic Properties of the B. Coli.—In man, *B. coli* has been found as the only organism present in various suppurative conditions (see Chapter VII.), especially in connection with the intestine (*e.g.* appendicitis) and about the urinary tract. In the latter, it is also responsible for catarrhal conditions in the pelvis of the kidney and in the bladder, these being more common in the female, and frequently presenting chronic characters. *B. coli* infection of the pelvis of the kidney

may be the sequel to cystitis, *i.e.* an ascending infection, but cases are not infrequent in which the organism has reached the kidney either by the blood stream or, as has been suggested, by communicating lymphatic channels from the bowel.

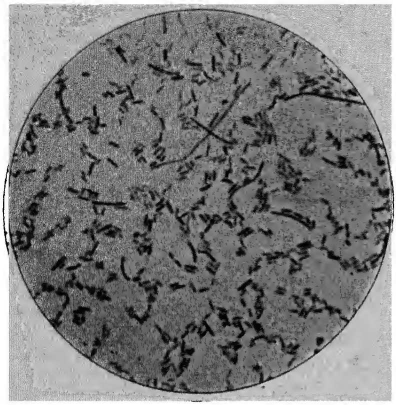


FIG. 109.—Typhoid bacilli, from a young culture on agar, showing some filamentous forms.
Stained with weak carbol-fuchsin. ×1000.

Many interesting problems arise in regard to the pathogenicity of *B. coli*. Under ordinary conditions this organism exists as a harmless commensal in the bowel of all the mammalian animals, and it is present usually in enormous numbers. No injurious influence is exerted normally on the intestinal mucosa. It seems probable, however, that small numbers may in the healthy person gain access to the lymphatics and even the blood stream; these are rapidly eliminated, but in certain conditions of lowered resistance may become established in certain tissues, *e.g.* the kidney. Obviously the healthy intestinal mucosa presents a high degree of local immunity to these organisms, whereas other tissues are less resistant.

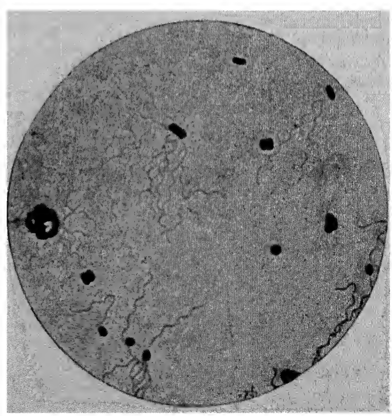


FIG. 110.—Typhoid bacilli, from a young culture on agar, showing flagella. See also Plate III., Fig. 16.
Stained by Kirkpatrick's method. ×1000.

The question also arises as to the rôle of *B. coli* in various pathological conditions of the intestine initiated by specific pathogens, e.g. *B. typhosus*, *B. dysenteriæ*, etc. Sanarelli found that *B. coli.* isolated from

typhoid stools was much more virulent for laboratory animals than when isolated from the stools of healthy persons. Coliform bacilli probably play the part of secondary infecting organisms when the mucous membrane is damaged or ulcerated by some other agent, *e.g.* in typhoid fever, in amœbic and bacillary dysentery, etc. Certain types may be more active in this respect than others. Attention has been drawn to the fact that in bacillary dysentery concomitant or secondary infections occur particularly with various non-lactose-fermenting strains, *e.g.* Morgan's bacillus and related organisms, paracolon bacilli (*vide infra*).

BACILLUS TYPHOSUS
(*Eberthella typhi*)

Microscopical Appearances.—As observed in pure culture and in the tissues, the individual organisms are straight, cylindrical or rod-shaped structures, with rounded ends, measuring on an average 2–4 μ long and 0·5 μ in thickness. Frequently in culture filamentous forms are noted, even 10 μ or more in length. In culture the bacilli occur singly and in pairs end to end. In tissues, *e.g.* in a Peyer's patch, spleen, liver, or mesenteric gland, they are found in relatively large clumps (*vide* Fig. 111).

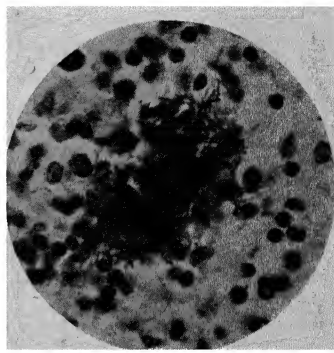


FIG. 111.—A large clump of typhoid bacilli in a spleen. The individual bacilli are only seen at the periphery of the mass. (In this spleen enormous numbers of typhoid bacilli were shown by cultures to be present in a practically pure condition.) Paraffin section; stained with carbol-thionin. ×500.

For their demonstration in tissue sections, carbol-thionin may be used (*vide* p. 116), or prolonged staining with Löffler's methylene-blue (half an hour), the sections being dehydrated and cleared with aniline-xylol (*vide* p. 112). The typhoid bacillus is Gram-negative.

Motility.—In hanging-drop preparations the bacilli are found to be actively motile. The smaller forms have a darting or rolling motion, passing quickly across the field, while some show rapid rotatory motion. The filamentous forms have an undulating or serpentine motion, and move more slowly.

Hanging-drop preparations should be made from agar or broth cultures, preferably within six to twelve hours after incubating at 37° C. and not more than twenty-four hours old. In older cultures the movements are less active.

Flagella.—On being stained by the appropriate methods (*vide* p. 124), the bacilli are seen to possess many long wavy flagella which are attached all along the sides and to the ends, *i.e.* peritrichous (Fig. 110).

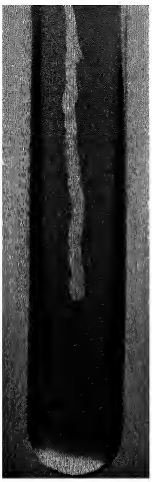


FIG. 112.—Stroke culture of *B. typhosus* on agar, twenty-four hours' growth.

Appearances of Cultures.—To grow the organism artificially from cases *post mortem* it is best to isolate it from the spleen (for method, see p. 169), or from the bile in the gall-bladder, as it exists in these situations in greater numbers than in the other organs, and may be the sole organism present even some time after death. Plates of MacConkey's medium may be employed (*vide* p. 73). The obtaining of pure cultures from cases during life will be dealt with in detail later (p. 555). On culture media the growths are visible after twenty-four hours' incubation at 37° C. On MacConkey's medium the colonies are small and colourless (cf. *B. coli*).

Generally speaking, on artificial media growths of the *B. typhosus* appear less dense than those of the *B. coli*. In stroke cultures on *agar* there is a greyish film of growth, with fairly regular margins, but without any characteristic features (Fig. 112). This film is moist, loosely attached to the surface, and can be easily scraped off. Colonies after twenty-four hours' incubation are small, semi-transparent, circular discs, but if growth is allowed to continue for a few days they enlarge considerably, become film-like with a wavy margin, a raised centre, and often radial ridges; this type of colony has been likened to a vine leaf. A certain amount of variation, as regards details of structure, may be observed among different strains. Stab cultures in *gelatin* yield the following appearance: on the surface of the medium growth spreads outwards from the puncture as a thin leaf-like film, with irregularly wavy margin; it is semi-transparent and of bluish-white colour; ultimately this surface growth may reach the wall of the tube; not infrequently, however, the surface growth is less marked;

along the stab there is an opaque whitish line of growth, of finely nodose appearance; there is no liquefaction of the medium (Fig. 113). On gelatin, surface colonies are more transparent than those on agar but present similar features.

Arkwright has found that certain strains of *B. typhosus* and other members of the group—*B. paratyphosus*, *B. dysenteriæ*, *B. enteritidis* (Gaertner), etc.—may show two types of colonies on plates, these being smooth (S) and rough (R) respectively (Fig. 114). Growths from the R colonies are spontaneously agglutinable in normal saline (though forming permanent suspensions in weaker saline) and form in broth a granular sediment with clear fluid and sometimes a surface scum in these respects differing from the S growths, which give a uniform turbidity. The two types differ also in their antigenic structure and their agglutination reactions (*vide* p. 234). These properties tend to be maintained in subcultures, but the S type may yield R-variant colonies on continuous cultivation.

Fig. 113. — Stab culture of *B. typhosus* in gelatin, five days' growth.

Growth on Potato.—For several days (at incubator temperature) after inoculation there is apparently no growth. If looked at obliquely, the surface appears wet, and if it is scraped with the platinum loop, a glistening track is left: a film preparation shows numerous bacilli. Later, however, a slight pellicle with a dull, somewhat velvety surface may appear. These appearances are only seen when fresh potato has been used.

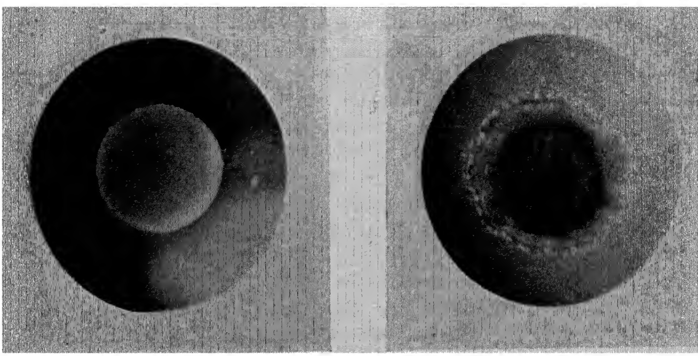
MacConkey's bile-salt neutral-red lactose agar. — Colonies resemble those on ordinary agar. They remain colourless or "pale" as compared with those of *B. coli* (*vide* p. 512), owing to the absence of lactose fermentation.

Conditions of Growth, etc.—The optimum temperature of the typhoid bacillus is about 37° C., though it also flourishes well at room temperature. It will not grow below 15° C., or above 41° C. Its powers of resistance correspond with those of most non-sporing bacteria. It is killed by exposure for ten minutes at 56° C., or almost instantaneously at 100° C. Typhoid bacilli kept in distilled or in ordinary tap water have usually been found to be dead after three weeks.

Biological Reactions.—The growth of the typhoid bacillus

on certain special media facilitates its being differentiated from the *B. coli* and the other members of the coli-typhoid group by biochemical reactions (see Table, p. 556).

The tests with sugars are important. The typhoid bacillus produces acid *without gas* in glucose, lævulose, galactose, xylose, dextrin, maltose and mannitol, but originates no change in lactose, saccharose or rhamnose. In arabinose and dulcitol late acid formation has been observed. On media containing these carbohydrates colonies may develop papillae which differ from the parent strain in fermenting the particular substances; these are analogous to the lactose-fermenting



A B

FIG. 114.—A, smooth, and B, rough type of colony of *B. typhosus*. ×20.

papillæ of *B. coli mutabilis* (*q.v.*). There is no curdling of milk, although at first slight acid production occurs; in a variable time the acid change may be succeeded by a neutral reaction. The typhoid bacillus is incapable of producing indole in peptone solution, and does not alter neutral red in lactose broth. It forms hydrogen sulphide in culture medium (p. 535). Salts of organic acids are not utilised by this organism.

Bacillus typhosus contains characteristic somatic (O) and flagellar (H) antigens, and immunisation with the organism leads to the formation of agglutinins which react specifically with these antigens. It may be noted here that the somatic antigen of the typhoid bacillus is identical with that of *Bacillus enteritidis* and certain other members of the *Salmonella* group (p. 544). The flagellar antigen also is common to certain

Salmonella types (*e.g.* Stanley, *vide infra*), but these possess different somatic components. The identity of the typhoid bacillus can therefore be conclusively established by means of agglutination reactions. Thus, if the serum of an animal immunised with a known strain of *B. typhosus* agglutinates an organism with the cultural and biochemical characters of this species, to the same end-titre as the known strain, the organism in question may be identified almost certainly as the typhoid bacillus. Further reference will be made later to the serological differentiation of the typhoid bacillus from allied types.

Freshly isolated strains of *B. typhosus* which are more virulent to mice than stock cultures, possess an additional somatic antigen which has been designated " Vi " in virtue of its association with virulence (Felix). The presence of this antigen seems to render the organism relatively inagglutinable by an O antiserum, but such inagglutinability disappears on subculture in parallel with the lose of the Vi antigen. This antigen is destroyed by ten minutes' exposure at 100° C.; it is also partially destroyed by phenol. Strains produce it best when growing in a soft ascitic-fluid agar. A pure Vi antiserum can be obtained by immunisation with a living culture known to contain the Vi antigen and by then absorbing from the serum H and O agglutinins with strains containing only the corresponding antigens; such a treated serum produces granular agglutination, which may be regarded as due to the interaction of the Vi antigen with its appropriate antibody. It has been claimed that the immunising properties of a typhoid vaccine depend to a considerable extent on the presence of this antigen in the strain selected and that the therapeutic action of a typhoid antiserum is likewise dependent on the presence of the Vi antibody (*vide infra*).

Invisible Form of Typhoid Virus.—According to Friedberger and his co-workers, the typhoid bacillus from post-mortem material, when passed in series through guinea-pigs, soon assumes an invisible form which is incapable of being cultivated on ordinary media. The evidence for the existence of this virus is that guinea-pigs which have been inoculated with it develop pyrexia, and thereafter are resistant to inoculation with virulent typhoid bacilli; further, the intravenous injection into rabbits of a suspension of the organs of such guinea-pigs taken during the pyrexial period leads to the development of agglutinins for *B. typhosus*.

The Pathology of Typhoid Fever.[1]—The inflammation and

[1] In the succeeding sections dealing with the pathology of typhoid fever, the pathogenesis of the typhoid bacillus, carriers, and epidemiology, most of the facts recorded apply also to the paratyphoid bacilli (described later); enteric fever is a convenient general designation for both typhoid and paratyphoid infections.

ulceration in the *Peyer's patches and solitary glands of the small intestine* are important features. In the early stage there is an acute inflammatory condition, and infiltration with mononuclear cells, sometimes attended with small hæmorrhages. At this period the typhoid bacilli are most numerous in the patches, groups being easily found between the cells. The subsequent necrosis may be due to the action of toxic products of the bacilli; the latter, however, gradually disappear, though they may still be found in the deeper tissues and at the spreading margin of the necrosed area. They also occur in the lymphatic spaces of the muscular coat. The number of the ulcers arising in the course of a case bears no relation to its severity. Small ulcers may occur in the lymphoid follicles of the large intestine.

The *mesenteric glands* corresponding to the affected part of the intestine are usually enlarged, sometimes to a very great extent, the whole mesentery being filled with glandular masses. In such glands there may be acute inflammation, and occasionally patchy necrosis occurs. An outstanding feature of the lesions is the extensive proliferation of the endothelial cells along the lymph sinuses and paths, attended by aggregations of mononuclear leucocytes. In other words, the reaction is of the *macrophage* type, and few polymorphonuclear leucocytes are present as a rule. Sometimes on section the glands are of a pale-yellowish colour, the contents being diffluent and consisting largely of leucocytes. Typhoid bacilli may be isolated both from the glands and the lymphatics connected with them, but *B. coli* is in addition often present.

The *spleen* is enlarged, usually of a fairly firm consistence, of a reddish-pink colour, and in a state of congestion. Of all the solid organs it usually contains the bacilli in greatest numbers. They can be seen in sections, occurring in clumps between the cells, there being no evidence of local reaction round them (Fig. 111). Similar clumps may occur in the *liver* in any situation, and without any local reaction. In this organ, however, there are often small foci of leucocytic infiltration, in which bacilli may not be demonstrable. The bacillus is found, often in large numbers, in the gall-bladder, where, in recovered cases, it may persist for years (*vide infra*). Clumps of bacilli may also occur in the *kidney*, and localisation of the organism may be observed in the *bone-marrow*.

In addition to these local changes in the solid organs, there are also widespread *cellular degenerations* in the solid organs, which suggest the action of toxic products.

In the *lungs* there may be bronchitis, patches of congestion and

of acute broncho-pneumonia. In these, typhoid bacilli may sometimes be observed, but evidence of a toxic action depressing the powers of resistance of the lung tissue is found in the fact that the pneumococcus frequently occurs in such complications of typhoid fever.

The *nervous system* shows little change, though meningitis associated either with the typhoid bacillus, with *B. coli*, or with streptococci has been observed.

During the first seven to ten days of the illness, and also in relapses, the bacilli can be isolated readily from the *blood*. They have been found in the *roseolar spots* which occur in typhoid fever, but it cannot be yet stated that such spots are always due to the presence of the bacilli. The fact that the typhoid bacilli are usually confined to certain organs and tissues shows that they probably have a selective action.

The reaction of the body to the typhoid bacillus is markedly one of the lymphoid tissues. This is further indicated by the blood changes, for, while there is a leucopenia, the lymphocytes are relatively increased in numbers. A general reaction is manifested by the appearance of bactericidal and other antibodies in the serum.

Course of Infection.—Infection takes place by ingestion of the organisms and their passage into the small intestine where they invade the tissues and lymphatics and so reach the general circulation, producing the bacteriæmia which is a characteristic feature of the early stage of the disease, *i.e.* during the first seven to ten days. From the blood they localise in the liver, spleen, kidneys, bone-marrow, and other tissues. As immunity reactions develop the bacteriæmia declines. From the liver capillaries and bile canaliculi the typhoid bacilli reach the gall-bladder and multiply in the bile, and at this stage large numbers are discharged into the intestine and appear in the fæces. This is most pronounced in the third week of the illness. As will be shown later, the organism may persist in the gall-bladder and sometimes in other tissues after apparent recovery.

Suppurative lesions occurring in connection with Typhoid Fever.—In a certain proportion of such lesions the typhoid bacillus has been the only organism found. This has been the case in subcutaneous abscesses, in suppurative periostitis, suppuration in the parotid, abscesses in the kidneys, empyema, etc., and also in a few cases of ulcerative endocarditis; suppuration due to the typhoid bacillus may be of a very chronic and recurrent nature. In the majority of cases other organisms, especially *B. coli* and pyogenic micrococci, have been obtained, the typhoid bacillus having been searched for in vain. It has,

moreover, been experimentally shown, notably by Dmochowski and Janowski, that suppuration can be experimentally produced by injection in animals, especially in rabbits, of pure cultures of the typhoid bacillus, the occurrence of suppuration being favoured by conditions of depressed vitality, etc. These observers also found that when typhoid bacilli were injected along with pyogenic staphylococci, the former died out in the pus more quickly than the latter. Accordingly, in clinical cases where the typhoid bacillus is present alone, it is improbable that other organisms have been present at an earlier date.

Occurrence of Gall-stones in those who have suffered from Typhoid Fever.—As has been stated, foci of bacilli occur in the liver in typhoid fever, and infection of the gall-bladder is a frequent feature of the disease. In the gall-bladder they may set up a catarrhal process (*cholecystitis typhosa*), though frequently they produce comparatively little change in the mucosa. There is evidence that the bacilli may persist in the gall-bladder for many years, and the catarrhal inflammation which they keep up leads to the formation of gall-stones. *B. coli* may be superadded. Typhoid bacilli have been isolated from cases of gall-stones operated on years after an attack of typhoid fever, and the bacilli have even been found within the calculi. They have also been demonstrated in chronic suppuration occurring in the gall-bladder.

Pathogenic Effects produced in Animals by the Typhoid Bacillus.—There is no disease of animals which is identical with typhoid, nor is there any evidence of the occurrence of the typhoid bacillus under ordinary pathological conditions in the bodies of animals. Attempts to communicate the disease to animals by feeding them on typhoid dejecta have been unsuccessful, and though pathogenic effects have been produced by introducing pure cultures in food, the disease has usually borne no resemblance to human typhoid. The results of subcutaneous or intraperitoneal injection are no more satisfactory. The type of disease is very different from what occurs naturally in man, and is often merely an acute toxæmia due to the endotoxin present in the organisms. In such injection experiments the results vary considerably—no doubt due to the fact that different strains of the bacillus vary much in virulence, ordinary laboratory cultures being often almost non-pathogenic. Reference has been made above (p. 521) to the virulence for mice of recently isolated strains containing the Vi antigen. Certain workers using highly virulent strains have been able by intraperitoneal injection to produce in animals effects on the

Peyer's patches, mesenteric glands, and spleen similar to those in the disease in the human subject. It has also been found by experiments in rabbits in which recently isolated cultures of *B. paratyphosus A* were introduced directly into the gall-bladder, that a pathological picture corresponding to that of the natural disease could be reproduced, with characteristic effects on the lymphoid patches in the intestine, the mesenteric glands and the spleen. Metchnikoff and Besredka claimed to have reproduced in monkeys a condition similar to human typhoid fever by feeding the animals with typhoid bacilli. It is specially noteworthy that in rabbits injected intravenously with typhoid cultures, the bacilli may become localised in the gall-bladder, where they persist as in the human carrier. According to Coplans, the bacilli may also localise in the bone-marrow after intravenous injection and persist there after they have disappeared from other parts of the body.

The Toxic Products of the Typhoid Bacillus.—There exist in the bodies of typhoid bacilli toxic substances which in artificial cultures do not pass to any great degree out into the surrounding medium; unlike the exotoxins they have no specific effects. The bodies of bacteria killed by chloroform vapour are very toxic—more so than filtered cultures—and there is evidence of the release of poisons from the organisms when these undergo lysis in the animal body. Macfadyen, by grinding up typhoid bacilli frozen solid by liquid air, produced a fluid whose toxic effect he attributed to the presence of the intracellular poisons.

Light has been thrown on the chemical nature of the toxic products of the group of organisms to which the typhoid bacillus belongs, by studies of chemical fractions isolated from *B. aertrycke* and *B. enteritidis* (p. 540). Such work has indicated that the toxic principle of these organisms is a complex carbohydrate combined with phosphatide, this substance being also antigenic (Raistrick and Topley).

The Immunisation of Animals against the Typhoid Bacillus.—Earlier observers had been successful in immunising mice to the typhoid bacillus by the successive injections of small and gradually increasing doses of living cultures of the bacillus. Later, Brieger, Kitasato, and Wassermann found that the bacillus, when modified by being grown in a broth made from an extract of the thymus gland, no longer killed mice and guinea-pigs. These animals after injection were moreover immune, and it was also found that the serum of a guinea-pig thus immunised could, if transferred to another guinea-pig,

protect the latter from the subsequent injection of a dose of typhoid bacilli to which it would naturally succumb. Chantemesse and Widal, Sanarelli, and also Pfeiffer, immunised guinea-pigs against the subsequent intraperitoneal injection of virulent living typhoid bacilli, by repeated and gradually increasing intraperitoneal or subcutaneous doses of dead typhoid cultures in broth. Experiments performed with serum derived from typhoid convalescents indicate that it has similar *anti-bacterial* powers, but there is no evidence that it contains any *antitoxic* bodies (see chapter on Immunity). Pfeiffer, for example, found on adding serum from typhoid convalescents to typhoid bacilli killed by heat, and injecting the mixture into guinea-pigs, that death took place as in control animals which had received these toxic agents alone. Pfeiffer also found that by using the serum of immunised goats, he could, to a certain extent, protect other animals against the subsequent injection of virulent living typhoid bacilli. On trying to use the agent in a curative way, *i.e.* injecting it only after the bacilli had begun to produce their effects, he obtained little or no result.

General View of the Relationship of the B. Typhosus to Typhoid Fever.—1. Typhoid fever is a disease in which the lesions are centred in the intestine, but the infection is essentially a general and systemic one, and secondary effects may occur in other parts of the body. The specific association of the typhoid bacillus with the disease and its characteristic lesions has been thoroughly estalished by bacteriological investigation. Its almost constant presence in the blood at an early stage is specially significant of its etiological relationship.

2. The comparative failure of attempts to cause the disease in animals is of little significance. In nature animals do not suffer from this condition, and laboratory animals are also insusceptible to the infection as it occurs in man.

3. The observations on the protective power against typhoid bacilli shown to belong to the serum of typhoid patients and convalescents, and the action of such serum in agglutinating the bacilli (*vide infra*), indicate an etiological relationship between the bacillus and the disease. Additional evidence is found in the fact that vaccination with the dead bacilli (*vide infra*) has a marked effect in preventing the disease from arising in those exposed to infection, and also in lowering the mortality when the fever attacks vaccinated persons.

These facts constitute indirect but practically conclusive evidence of the causal relationship of the typhoid bacillus to the disease. Confirmation of this view is found in the fact that cases have occurred where bacteriologists have accidentally infected themselves by the mouth with pure cultures of the typhoid bacillus, and after the usual incubation period have developed typhoid fever. Several cases of this kind are on record and their significance is not affected by the fact that other similar instances have occurred without the subsequent development of illness. These latter would

be accounted for by a low degree of susceptibility on the part of the individual or to a lack of pathogenicity in the cultures.

There is evidence that certain individuals are relatively insusceptible to typhoid fever. The cases of the occurrence of typhoid bacilli in the healthy intestine support this view, and it has been further shown that during an epidemic certain persons may suffer from slight intestinal symptoms with typhoid bacilli in the fæces without the disease going through its usual course. The so-called "ambulatory" cases of typhoid fever form a link between these mild infections and fully developed typhoid fever.

Typhoid Carriers.—In the great majority of cases of typhoid fever, the bacilli disappear from the fæces within from two to ten weeks of convalescence, but in a certain proportion of cases, probably about 2 to 5 per cent., evidence is found of the persistence of the bacilli for many months, and in certain cases their existence has been demonstrated even thirty years after the attack of illness. Carriers have been arbitrarily classified as "temporary" (*i.e.* those excreting bacilli up to a year after an attack of fever) and as "chronic" (those in whom this period is exceeded), but the distinction is unimportant. It may be said that the majority of carriers to whom outbreaks have been traced are women. Persons in whom the carrier state is present are a constant danger to those around them, as the infectivity of the bacilli frequently remains, and during recent years the importance of such carriers has been recognised as explaining many outbreaks of the disease. The cases traceable to such an origin are of the type usually classed as sporadic. They arise among persons associated with carriers, especially when the latter are concerned in the preparation of food. From time to time, however, large epidemics have arisen from a carrier having contaminated a milk supply in a dairy. The site of the multiplication of the bacteria in a great many of these carriers is probably the gall-bladder. As has been stated, the typhoid bacilli may persist there for many years, often giving rise to gall-stones. An additional danger lies in the fact that carriers usually appear to be in perfect health or may only suffer from slight manifestations of cholecystitis and biliary calculus—it is well known that in only a proportion of patients suffering from gall-stones do severe symptoms arise. An additional factor in the carrier problem lies in the fact stated above, that apparently when certain persons ingest the typhoid bacilli, the latter may establish themselves in the body without giving rise to typhoid fever. Such persons have been referred to as "paradoxical" carriers; they represent those who either are naturally insusceptible to typhoid fever

or have developed immunity in consequence of a previous attack. The most serious danger to a community arises, however, from the "chronic" carrier. In certain carriers, the focus of multiplication of the typhoid bacillus may be the kidney, the bacilli in such cases passing out in the urine. Urinary carriers are much less common than intestinal carriers; but, unlike the latter, they occur equally in the two sexes.

The tracing of a typhoid carrier constitutes an important and difficult problem. Firstly, the serum of all suspicious persons ought to be submitted to the Widal test (*vide infra*). Usually speaking, the carrier gives a positive reaction, but sometimes this is absent and sometimes is only obtained with a high concentration of the serum. Further, it has been shown in chronic carriers that the agglutinating capacity of the serum varies from time to time and sometimes may be absent. The proof of a person being a carrier lies essentially in the isolation of the typhoid bacillus from the fæces or the urine, and it is to be noted that, especially in the former, the organism is not constantly present—in certain cases months of remission have been recorded. Several explanations have been advanced to account for the facts observed, such as the occurrence of symptomless re-infections or of periodic more or less acute auto-infections from a latent focus of persistence of the organism, *e.g.* in the gall-bladder. In any case, the necessity for repeated investigation of a suspected carrier is obvious. The methods to be adopted are detailed on p. 558. Much work has been directed to the question of freeing the typhoid carrier from the organism; various methods, such as intestinal antisepsis, vaccination, excision of the gall-bladder, have been tried. Hitherto success has not been obtained except as a result of operative procedures (excision of the gall-bladder and drainage of the ducts), which appear to have cured a considerable proportion of intestinal carriers. In certain urinary carriers examination of the urine from each ureter has shown that the infection is unilateral, being maintained by an abnormal condition such as a calculus in the ureter or kidney pelvis. From the public health standpoint, the prevention of the presence of carriers in a population must be provided for; and in fever hospitals means are taken for retaining convalescents from typhoid until the bodily discharges are free from the typhoid bacillus.

The Epidemiology of Typhoid Fever.—In civilised communities the prevalence of typhoid fever has been very markedly reduced, coincident with the substitution of central filtered water supplies for well water and with the improvements

effected in general sanitation and especially in the removal of excreta and refuse. In certain localities, however, periodic outbreaks, often of a seasonal character, still occur. At one time these were attributed to the capacity of the typhoid bacillus to live and multiply outside the human body. There is, however, no evidence that the typhoid bacillus can maintain a saprophytic existence though it may remain viable in sewage, water, etc., for a variable time. Thus *B. typhosus* and *B. paratyphosus B* have been demonstrated by methods of selective cultivation in the sewage of large communities. In water it may survive for at least several days, and even for two or three weeks, the time depending on the degree of contamination of the water with other organisms. Thus typhoid bacilli tend to persist longer in a relatively pure water than in one grossly polluted and containing large numbers of putrefactive organisms. The existence of carriers in all communities where typhoid fever occurs has, however, thrown light on the subject and has accounted for the origin of outbreaks. In many cases survival outside the body for some time is an essential factor where a water or food supply becomes infected with material derived from a carrier, but direct and mediate contact infection also plays a great part in the incidence of the disease. At the present time small outbreaks frequently originate in those who are brought into domestic contact with carriers, and larger epidemics may occur when a carrier pollutes a water or especially a milk supply. During such epidemics secondary cases may also arise from contact with primary cases.

It is now well known that the house-fly and certain other filth-feeding insects may act as vectors of typhoid bacilli, and contaminate food to which they gain access after having been in contact with human excreta. Such convection is most marked under conditions of imperfect sanitation, in which human excretal matter is freely exposed to flies, particularly in tropical and subtropical countries and also in the warm season of temperate climates when flies are most numerous. This affords some explanation of the seasonal incidence of enteric fever, its prevalence in ill-sanitated areas and its incidence under war conditions, particularly in campaigns waged in hot climates.

The fly may carry excretal bacteria on the surface of its body, wings, and legs, but typhoid bacilli may also persist in the alimentary tract of the insect after ingestion of infective excreta (Graham Smith and others). Food is thus contaminated by the insect as a result of its regurgitation and defæcation. In a

series of experiments, in which captive flies were artificially infected with typhoid and paratyphoid bacilli and after varying intervals allowed to walk about on a plate of MacConkey's medium which was then incubated, we have found that they remained infective for as long as six days. Colonies of the bacilli developed on the plate in circular groups suggestive of their originating from defæcated or regurgitated material.

Occasional outbreaks of enteric fever and sporadic cases have been traced to oysters and other shellfish, uncooked vegetables such as water-cress, lettuce, etc., contaminated from sewage or human excreta. It has been shown that typhoid bacilli may persist in living oysters for a considerable time.

The Serum Diagnosis of Typhoid Fever—Widal Reaction.—While the most conclusive method of establishing the diagnosis of a typhoid infection is the isolation of the organism (*vide* p. 555), the indirect proof of the infection by serum diagnosis has been extensively used in routine laboratory work. The method of carrying out this test has been described on p. 138. It depends on the presence in the patient's blood serum of specific agglutinins for the typhoid bacillus. The macroscopic method is generally used, and the test is carried out on a strictly quantitative basis so that the highest dilution in which agglutination occurs, *i.e.* the " end-titre," can be determined. In the diagnosis of typhoid fever, paratyphoid infections have also to be considered and parallel tests are carried out with the paratyphoid bacilli (*vide* p. 535). Owing to the fact that normal serum in high concentrations may agglutinate the typhoid bacillus, it is necessary to base the diagnostic interpretation of results on the known range of such " normal serum effects " ; if, however, marked agglutination occurs in a dilution of 1 : 60 with average cultures of *B. typhosus* by the macroscopic method in a person not vaccinated against the typhoid bacillus, the result may be regarded as diagnostic.

All strains do not give uniformly the same results, though it is not definitely known on what this difference of susceptibility depends. A strain must therefore be selected which gives the best results in the greatest number of undoubted cases of typhoid fever, and which gives as little reaction as possible with normal sera or sera derived from other febrile diseases. This latter point is important, as some strains react very readily to non-typhoid sera. Again, care must be taken as to the *state of the culture* used. The suitability of a culture may be impaired by varying the conditions of its growth. Dreyer's standardised bacillary suspensions are of great value in view

of these variations (p. 140). Their preservation, however, by formalin introduces a difficulty in their use. Formalin interferes with O agglutination, though the " large flake " agglutination characteristic of the H type of antigen is well marked with formolised suspensions. Both O and H agglutinins are formed in typhoid infections, but in some cases the agglutinins may be mainly of the O type, and the formolised suspensions may then give negative results. These suspensions are, however, suitable for testing the H agglutinin. An analogous standard suspension containing the O antigen alone can be prepared by treating cultures with alcohol (*vide* p. 141), the H antigen being annulled by this method. The use of both formolised and alcoholised suspensions has therefore been recommended as a means of testing qualitatively for H and O agglutinins. Felix has advocated for the purpose specially selected strains which are sensitive to the H and O agglutinins respectively, and formolised and alcoholised standard suspensions from these should be utilised. In carrying out the routine Widal test many workers have now adopted the practice of employing, in parallel, standard H and O suspensions of *B. typhosus*. In the diagnostic interpretation of reactions obtained with these suspensions normal serum agglutination must be taken into account. According to the Oxford Standards Laboratory, titres below the following limits cannot generally be considered significant whereas the probability of significance increases from the limit upwards: *B. typhosus* (H), 1 in 30; (O) 1 in 50. Those figures refer to standard titres obtained by the method recommended by the Standards Laboratory. It has been found in this country that normal serum agglutination may occasionally reach higher titres, *e.g.* with H suspensions 1 in 80, and with O suspensions, 1 in 100. The further application of these methods is discussed later (p. 536).

The reaction given by the serum in typhoid fever usually begins to be observed about the seventh day of the disease, though occasionally it has been found as early as the fifth day; sometimes it may be delayed. Usually it becomes gradually more marked as the disease advances, and it is still given by the blood of convalescents. Further, it may persist for several months or longer after recovery. As a rule, up to a certain point, the reaction is more marked where the fever is of a pronounced character, while in the milder cases it is less pronounced, but this is not invariably the case.

Owing to the fact that the development of the specific agglutinins is progressive during the illness, if, on first testing, agglu-

tination is absent or occurs only in low dilutions within the normal agglutination range, the test should be repeated. In this way a more conclusive result may be obtained. A series of tests carried out at intervals may elicit a " rising " end-titre which is, of course, of the highest diagnostic significance.

In cases previously vaccinated with the typhoid bacillus, a serious fallacy may be introduced into the ordinary diagnostic test when applied to such persons developing an illness suspected to be a typhoid infection. Thus in healthy persons vaccinated seven to fourteen months previously, the serum may agglutinate the typhoid bacillus in dilutions of 1 : 200 and even in some cases in higher dilutions (Martin and Upjohn). It is of interest to note that in those who have been vaccinated with the typhoid bacillus, a subsequent paratyphoid infection may lead to an increase in the " post-vaccine " typhoid agglutinin, and high-titre reactions may occur with the typhoid bacillus as well as with the causal organism (Mackie and Wiltshire). Other infections, *e.g.* various febrile illnesses, may produce a similar effect. These questions will be referred to in more detail later (p. 536).

Vaccination against Typhoid.—The principles of the immunisation of animals against typhoid bacilli were originally applied by Wright and Semple for prophylactic purposes. The method of preparing the vaccine has been described on p. 158. Two doses have usually been given, separated by an interval of ten days, the first consisting of 500 million bacilli and the second of 1000 million. (A typhoid-paratyphoid vaccine is now generally used, containing *B. typhosus*, *B. paratyphosus A* and *B*, and is frequently designated " T.A.B."—*vide* p. 536). The effects of the first injection are some tenderness locally, and in the adjacent lymphatic glands, and, it may be, local swelling, all of which come on in a few hours, and may be accompanied by general malaise and a rise of temperature, but the illness is transient. During the next ten days the blood of the individual begins to manifest, when tested, an agglutination reaction, and further, Wright found that usually after the injection there is a marked increase in the capacity of the blood serum to kill the typhoid bacillus *in vitro*. The second injection usually produces less marked symptoms, but is followed by a further increase of agglutinins in the serum. These observations indicate that the vaccinated person possesses a degree of immunity against the bacillus, a conclusion borne out by the results obtained in the use of the vaccine as a prophylactic against typhoid fever. During the late war the efficacy of

vaccine prophylaxis was put to the test on the most extensive scale. The incidence of enteric fever, an infection which has always been specially prevalent under war conditions, was minimal even in the campaigns waged in tropical and subtropical countries. In the early stages of the Mediterranean campaign, however, enteric fever was prevalent, but the great majority of the cases were paratyphoid infections, and it is significant that the troops were immunised at that time only against the typhoid bacillus. After the introduction of the combined typhoid-paratyphoid vaccine (*vide* p. 536), enteric infections generally were reduced to negligible proportions. It must be remembered that the duration of the artificial immunity is limited, and if it is necessary to maintain a constant prophylaxis, immunisation should be repeated at yearly intervals. It has often been recommended that vaccination should be carried out some time previous to the exposure to infection, on the view that immediately after inoculation there is a temporary lowering of resistance ("negative phase"). The possibility of such an effect has probably been overestimated. Recent work on the Vi antigen suggests the importance of preparing typhoid vaccine from a virulent strain containing this antigen.

The diminution in the incidence of typhoid fever in certain countries in recent times, both in war and peace, has been of a remarkable nature. It must be borne in mind that, in addition to antityphoid inoculation, the identification and isolation of carriers and also improvement in general sanitary conditions have been carried out on an extensive scale, and these measures must have contributed to the change in frequency of the disease. How far each of these factors has been individually responsible for the diminution it is not possible to say.

Antityphoid Serum.—Chantemesse immunised animals with dead cultures of the typhoid bacillus, and, having found that their sera had protective and curative effects in other animals, used such sera in human cases of typhoid with apparent good result. In the hands of others, however, such a line of treatment has not hitherto been equally successful. Recently good results have been recorded with an antityphoid serum containing Vi and O antibodies (Felix). It is claimed that the Vi antibody is highly active in promoting phagocytosis of virulent organisms containing the Vi antigen and that it excels the O antibody in this respect. These observations, however, require fuller confirmation.

The Isolation of the Typhoid Bacillus from Water Supplies and Sewage is dealt with in Chapter XXIX.

Paratyphoid Bacilli

In 1898 Gwyn recorded a case clinically resembling typhoid fever, and isolated from the blood the organism now designated *B. paratyphosus*. Since that time numerous outbreaks of enteric fever due to this organism have been observed. During the late war these came into great prominence from the fact that they constituted a prevalent group amongst intestinal infections as a whole. Clinically they may be indistinguishable from true typhoid, and under war conditions exhibited considerable severity, though they frequently take the form of a mild typhoid-like illness which may be transient. The intestinal lesions may be like those of typhoid fever, with or without ulceration, though notably less severe. There may also be peritonitis without perforation, and suppuration in brain, kidney, lymphatic glands, bones, etc. The old term " enteric fever " is now generally applied to all clinical cases of a typhoid type due to either the typhoid or paratyphoid organisms.

The paratyphoid bacilli have the general characters of the coli-typhoid group, motility being usually active though the flagella are often few in number. They are non-lactose-fermenters and produce acid and gas in glucose, mannitol, maltose, dulcitol, lævulose, galactose, sorbitol, and arabinose; they do not ferment raffinose, saccharose, salicin, or inulin. Of these reactions, that with lactose differentiates them from the common types of *B. coli*, and the production of gas distinguishes them from *B. typhosus* and *B. dysenteriæ*. They do not produce indole, though indole-forming variants have been described by certain observers. Two main types occur, denominated respectively *B. paratyphosus A* (*Salmonella paratyphi*) and *B. paratyphosus B* (*Salmonella schottmülleri*), the latter being the commoner in Western Europe, the *A* type being more prevalent in the East.

Cases of enteric fever have also been reported in various parts of the world as being due to a type of paratyphoid bacillus which is biologically different from the others, and this organism has been designated *B. paratyphosus C*. It is related serologically to *B. suipestifer*. Another type of paratyphoid bacillus, designated the " Sendai " type, has been observed in Japan. It is similar to the other paratyphoid bacilli in general characters, but differs in its antigenic structure. Certain further types referable to the *Salmonella* group, though differing in antigenic structure from the classical paratyphoid bacilli, have occasionally been recorded in cases of enteric fever, e.g. *B. suipestifer* (Kunzendorf type), Bareilly and Eastbourne types. These will be referred to later along with the food-poisoning organisms (p. 543).

The fermentative capacities of *B. paratyphosus A* and *B* are in general similar, but *A* is the less active—gas formation being often scanty and late in appearance. This is particularly noticeable in regard to dulcitol. In litmus milk *A* produces little or no change, while, in the case of *B*, there is rapid alkali production. A difference can also be established in regard to xylose and inosite fermentation and the formation of sulphuretted hydrogen in lead acetate agar (*vide* Table, p. 556). The *B* type utilises sodium citrate; *A* does not. These paratyphoid bacilli differ in their antigenic structure from one another and also from *B. typhosus*, and in the ultimate differentiation of the two types their reactions with specific agglutinating sera is of essential importance. They show certain serological relationships with other members of the *Salmonella* group, *e.g.* the organisms concerned in "food-poisoning," and this question is considered later (p. 543). Colonies of *B. paratyphosus B* frequently develop a striking character under certain conditions and in this respect differ from colonies of *B. paratyphosus A* and *B. typhosus*. This consists in the formation of an opaque raised margin developing when plates that have been incubated at 37° C. are subsequently allowed to stand at room temperature for twenty-four to forty-eight hours. This character, "schleimwallbildung" (Müller), may assist in the recognition of the colonies and the isolation of the organism from mixed growths (Fig. 115). Certain strains form smooth and rough colonies on agar plates, as has been described in the case of *B. typhosus* (Fig. 114).

FIG. 115.—Colonies of *B. paratyphosus B* after twenty-four hours' growth at 37° C. and forty-eight hours further at room temperature—showing the characteristic raised margin. ×1½.

What has been said with regard to pathogenesis, distribution in the body, viability, infection, carriers, epidemiology, and immunity in the case of the typhoid bacillus, is generally applicable also to the paratyphoid bacilli.

As in typhoid fever, patients suffering from paratyphoid develop specific agglutination properties in their blood serum

for the causative organism. Thus the serum of a patient infected with *B. paratyphosus A* may agglutinate this organism, but will have little or no effect on *B. typhosus* or *B. paratyphosus B*.

The principles applicable in the case of *B. typhosus* to the diagnostic use of the agglutination test also hold for the paratyphoid bacilli. Normal serum effects have to be specially considered in interpreting results, and quantitative tests should always be carried out in which a series of dilutions are tested by the macroscopic method (*vide* p. 139). In the case of *B. paratyphosus A*, marked agglutination in a dilution of 1 : 20 is significant, and this applies also to H and O suspensions. In the case of *B. paratyphosus B* normal serum agglutination may reach higher dilutions, and with average cultures (in the form of ordinary suspensions) an end-titre lower than 1 in 120 has not been considered significant. With standard H and O suspensions normal serum effects have been recorded in this country up to 1 in 40 and 1 in 80 respectively. The Oxford Standards Laboratory records the normal limit for a standard H suspension of *B. paratyphosus B* as 1 in 30 and for *B. paratyphosus A* 1 in 10. Further, such data refer only to persons unvaccinated against these organisms. Repeated tests may often be required to elicit conclusive results, and it is well known that in the paratyphoid *A* infections the agglutinin development is frequently weak or is almost absent till late in the disease.

It should be noted that normal agglutination may vary in degree in different parts of the world and among different communities. It is therefore important that those responsible for diagnostic Widal tests should be acquainted with the range of such normal reactions in the community in which they are working.

The methods of isolation of these organisms will be considered later (p. 555).

Preventive Inoculation.—All the evidence points to inoculation with *B. typhosus* having no effect in protecting against paratyphoid fever, and it is customary, in the prophylaxis of enteric fever, to use for the inoculation a mixture containing in the case of the first dose 500 million *B. typhosus*, 375 million *B. paratyphosus A*, and 375 million *B. paratyphosus B*, and after seven to ten days, as in the original method of typhoid vaccination, a second dose is given, containing double these quantities of the three organisms.

The Widal Reaction in persons previously inoculated with Typhoid-paratyphoid Vaccine.—Reference has been made (p. 532) to the

limitation of the Widal reaction in persons who have been inoculated against *B. typhosus* and later develop a suspicious illness. The same applies *a fortiori* when typhoid-paratyphoid vaccine has been administered and there is a possibility of either typhoid or paratyphoid infection. In order to surmount the difficulties arising from such complications some observers have used the method of making frequent—it may be daily—estimations during an illness of the highest dilutions in which the serum will agglutinate each of the organisms suspected to be the causal agent. This method was specially elaborated by Dreyer, Ainley Walker and Gibson, who held that the study of the curves of the agglutinin content of the serum gave valuable information. Thus, a regular and marked rise in the curve of one of the typhoid-paratyphoid subgroup to a maximum between the sixteenth and twenty-fourth day (especially between the eighteenth and twentieth) with a gradual fall thereafter, indicated an infection with that bacillus ; if in such a case primary agglutinins were present towards other members of the sub-group (due to a previous vaccination), the curves of these residual agglutinins either showed no change or a slight rise with a fall to their initial levels, or a marked rise, synchronous with or slightly earlier than that of the curve for the infecting organism. An attempt has also been made to overcome the difficulty by testing for H and O agglutinins separately by the methods referred to on p. 531, *e.g.* with formolised and alcoholised bacterial suspensions. It has been stated that in persons vaccinated some months previously the residual agglutinins are mainly of the H type, whereas in enteric fever both O and H agglutinins appear in the serum. Beattie and Elliot have found, however, in recent observations on naval ratings that seven to twelve months after inoculation with typhoid-paratyphoid vaccine the O agglutination with *B. typhosus* and *B. paratyphosus B.* may reach a serum dilution of 1 in 160. They conclude that only rarely could a diagnosis of enteric fever be made by a single O agglutination test.

Organisms associated with Food-Poisoning, and Allied Bacilli

Organisms biologically related to the paratyphoid bacilli are found associated with cases and outbreaks of acute enteritis following the ingestion of some article of food, frequently meat, contaminated with the particular organism. Such cases were at one time designated "ptomaine poisoning," from the idea originally prevailing that the symptoms were caused by ptomaine substances produced from the bacterial decomposition of the proteins of the food. The vast majority of cases of food-poisoning, taking the form of an acute enteritis, are due to the group of bacteria now under consideration, which are all capable of multiplying in the intestine ; they may also produce a general blood infection. (The condition of botulism, a special form of food-poisoning affecting the nervous system,

is discussed in Chapter XX.) The meat or food at fault may not, to taste or smell, present any unusual features, but very often there can be isolated from it an organism identical with that derived from the sick individuals. Sometimes it has been proved that the animals from which the food is derived have been suffering from infection due to the organism subsequently found, but this has not always been the case, and meat from healthy animals may be contaminated from extraneous sources. The foods giving rise to poisoning usually belong to the preserved-food class, *e.g.* sausages or similar products, but cases also arise from fresh foods, milk, etc.

Bacillus Enteritidis of Gaertner (*Salmonella enteritidis*).—The classical type of such organisms is *B. enteritidis* described first by Gaertner (1888) in an outbreak in Saxony of fifty-seven cases of gastro-enteritis following the ingestion of the flesh of a diseased cow. The organism was isolated both from the patients and from the meat. Subsequent similar outbreaks in Germany and in this country were found to be due to the same organism which was demonstrated in the stools and organs of cases. *B. enteritidis* also occurs in natural infections among rats. This organism resembles *B. paratyphosus B* in its general characters, including biochemical reactions (*vide* Table, p. 557), but can be clearly differentiated by agglutination reactions. Its antigenic constitution and relationships to other members of the *Salmonella* group are dealt with later (p. 543). The serum of convalescents may agglutinate the organism. *B. enteritidis* is very pathogenic for laboratory animals. Often, whatever the channel of infection, there is intense hæmorrhagic enteritis, and usually there is a septicæmia with the occurrence of serous inflammations; the bacilli are recoverable from the internal organs and often from the blood. In man, as the name of the bacillus indicates, the effects are centred in the intestine, where there is usually marked inflammation of the mucous membrane, sometimes attended with hæmorrhage into it; evidence of a septicæmic condition may also exist. Infection may take place by the bacillus itself, and here the illness usually appears within twenty-four hours of the food being partaken of, but symptoms may appear almost at once, in which case they are no doubt due to the immediate action of toxin; it is important to note that the poisons formed by this type of organism are relatively heat-resisting, so that boiling for a time does not destroy the toxicity.

Danysz's Bacillus and Rat Viruses.—Danysz isolated from an epizootic in field mice an organism which is practically identical

with *B. enteritidis*, and introduced it for the purpose of killing rats by originating in them through feeding a similar epizootic. Several viruses of this kind have been in commercial use for this purpose, e g. " Ratin." The efficacy of such agents may vary, and the mortality in artificially originated epizootics may be from 20 to 50 per cent. Sometimes, apparently under natural conditions, rats develop an immunity to such viruses.

Other Organisms associated with Food-Poisoning.— Since Gaertner's bacillus came to be recognised as a causal agent of " food-poisoning " of the type described, similar outbreaks in various countries have been carefully investigated bacteriologically, and other members of the *Salmonella* group have also been described as causal organisms. A great deal of confusion, however, has arisen in their classification and nomenclature. The *Salmonella* group considered as a whole comprises a number of types resembling one another in general and biochemical characters but serologically different. Even in serological characters, however, the group relationship among certain types is so close that agglutinin-absorption tests (*vide* p. 236) are necessary for their identification.

Savage and White in a study of the organisms of this group associated with food-poisoning, recognised in 1925 the following main types: *B. enteritidis* (Gaertner), *B. aertrycke*, *B. suipestifer*, and certain other varieties designated " Stanley," " Newport," etc. Recently a considerable number of other types have been reported (*vide infra*).

B. aertycke was originally isolated by De Nobele in an outbreak of food-poisoning at Aertrycke, in Belgium. *B. suipestifer* (or bacillus of hog cholera) had previously been described by Salmon and Theobald Smith in swine fever (or hog cholera), which, however, was later shown to be due to a filterable virus, *B. suipestifer* being probably a secondary agent in the disease. *B. aertrycke* and *B. suipestifer* had long been regarded as almost identical, and their association with food-poisoning was well recognised. The biochemical reactions of these organisms are shown in the Table on pp. 556, 557, and their antigenic characters in the Table on p. 544.

These organisms were at one time confused with *B. paratyphosus B* owing to their close serological relationship with it, and similarly strains designated *B. paratyphosus B* were described in food-poisoning cases. Savage and White separated these organisms into the different types referred to above, and pointed out that *B. paratyphosus B* is associated with paratyphoid fever only, and probably never occurs in food-poisoning. *B. suipestifer* they regarded as of exceptional occurrence in food-poisoning, though *B. aertrycke* is a frequent cause. Recent data on the occurrence of

Salmonella group organisms in human infections in this country indicate that *B. aertrycke* is by far the most prevalent. Other types which have been reported are *B. enteritidis* (including the *Dublin* variety), *Thompson*, *Newport*, *B. suipestifer* (diphasic and monophasic types), *B. morbificans bovis*, *Senftenberg* var. *Newcastle*, *Derby*, *Stanley*, *Eastbourne*, *Potsdam*, and *Aberdeen*. The serological characters of these organisms are dealt with below and their biochemical reactions are shown on p. 557.

Smith has emphasized the variation in the form of disease produced by *Salmonella* types: *B. aertrycke*, *B. enteritidis*, and the *Thompson* type are usually responsible for an enteritis, while the *Dublin* variety of *B. enteritidis* has been associated with septicæmia and meningitis. *B. suipestifer* also seems to possess considerable invasive properties. Cases have been recorded which at first had the symptoms of food-poisoning and later developed into enteric fever; it remains undecided whether such are due to mixed infections.

In the etiology of food-poisoning the sources of the causative organisms and the mode of contamination of the offending article of food require consideration. *B. enteritidis* occurs in natural infections in cows, calves, and rats. *B. aertrycke* is responsible for epizootic enteritis in guinea-pigs, mice, and other rodents, and is occasionally found in pigs, but not commonly in rats (Savage and White). Thus, in the case of flesh foods, the animal from which the food is derived may have been infected, as in the case of the original *B. enteritidis* strain. As noted above, preserved foods have frequently been responsible for outbreaks, and it has been assumed that growth of the particular organism may have occurred in the material with the formation of toxin which, on ingestion of the food, is responsible for immediate symptoms. As these organisms are prevalent in mice and rats, contamination of food may occur from these animals. *Salmonella* organisms, e.g. *B. aertrycke*, have been observed in infected birds, *e.g.* pigeons, ducks; the eggs from such birds may contain the organism, and food-poisoning has in some cases been traced to this source. In certain instances, human carriers of these organisms may be responsible for the food contamination and outbreaks of enteritis following the ingestion of the food.

Toxic and Antigenic Chemical Fractions isolated from Salmonella Group Organisms.—Studies of the chemical fractions isolated from *B. aertrycke* and *B. enteritidis* have been made by Boivin, Raistrick and Topley and others. By extraction of the bacterial cells and purification of the resulting products a protein-free fraction has been obtained consisting of a complex carbohydrate combined with phosphatide. This apparently constitutes the endotoxin and also the somatic antigen of the organism. It is highly toxic, and animals injected with it develop hyperglycæmia. *In vivo* it incites the production of somatic agglutinins. It has been suggested that this type of chemical substance isolated from *Salmonella* organisms is representative of similar toxic and antigenic products common to various bacteria and differing chemically from one another according to the species or type of organism.

Bacteriological diagnosis of Salmonella Food-poisoning.—Cultures should be made from the stools on plates of MacConkey's medium. Separate pale colonies are subcultured, and the cultures are investi-

gated first in regard to their biochemical characters and subsequently their serological reactions. In addition to the ordinary fermentation tests with sugars and similar carbohydrates shown in the Table on p. 557, certain other biochemical reactions have been advocated for differentiating between *B. paratyphosus B* and the food-poisoning organisms, *e.g. B. aertrycke*. Among these, two reactions merit attention as being of definite practical value: the occurrence or absence of growth on a medium containing ammonium chloride and rhamnose as the sources of nitrogen and carbon (Pesch). On this medium *B. aertrycke* grows while *B. paratyphosus B* fails to develop (for the composition of the medium reference should be made to the original article). The utilisation of sodium d-tartrate is also of differential value: thus *B. aertrycke* and various other *Salmonella* group organisms utilise this salt while *B. paratyphosus B* has no action on it (Brown, Duncan, and Henry). To test this difference the organisms are grown in peptone water containing 1 per cent. of the salt. After incubation a solution of lead acetate is added to the culture: if the salt has not been acted on there is a copious precipitate of an insoluble lead compound with the organic acid; utilisation is indicated by absence of such precipitation though a slight precipitate of carbonate may be observed. For fuller details of these reactions the original papers should be consulted. Careful agglutinin-absorption tests may be necessary before the strain can be identified with one of the recognised types. The serological identification of these organisms is considered below. In certain cases, particularly those of a severe nature, the causative organism is present in the blood and can be isolated by blood culture. The suspected article of food, if obtainable, should be examined bacteriologically with a view to isolating the causative organism from it. In convalescence, if a bacteriological diagnosis has not previously been made, examination of the patient's serum by direct agglutination tests and agglutinin absorption tests with known *Salmonella* strains of different types may elicit proof of the previous infection.

The Serological Classification of the *Salmonella* Group

In recent years the antigenic composition of the *Salmonella* group has been studied by various workers (notably by Bruce White and Kauffmann), and an attempt has been made to define in this way the serological differences and inter-relationships of the various types. Reference has been made to the close relationship between different members of the group and the difficulty of differentiating such organisms by simple agglutination tests with specific antisera. The occurrence of such cross-agglutination is attributed to the fact that an organism not only stimulates the production of agglutinins towards itself (primary or homologous agglutinins), but also of agglutinins acting on kindred species (secondary, heterologous, group agglutinins, or co-agglutinins). The primary agglutinins

are usually formed in much greater amount than the secondary; and careful quantitative tests usually elicit this difference. Among certain organisms group agglutination may be so marked as to render direct agglutination tests insufficient for differentiation.

In differentiating between primary and secondary agglutinins the absorption method (p. 141) is used. Thus, Castellani found that, when an animal had been immunised with *B. typhosus*, this organism would *in vitro* remove from the serum not only the primary typhoid agglutinins, but also the secondary agglutinins, which might act on allied organisms, whereas *in vitro* treatment (" absorption ") with a heterologous organism removed only the secondary agglutinins for that organism without influencing the primary agglutinins.

The occurrence of group agglutinins in the serum of an animal immunised against a particular organism is due to the complexity of the antigenic structure of the bacillus in question, some antigens being specific or peculiar to the bacillus, others being possessed in common with other members of the group. It was at one time assumed that the individual bacilli in a pure culture possessed the same antigenic structure. The work of Andrewes, however, showed that in the case of certain members of the *Salmonella* group the two kinds of antigen are carried by different bacilli and their derivative colonies in plate cultures. These organisms are therefore described as " diphasic." In analysing the subject, Andrewes first prepared a pure specific serum and a pure group serum. The former was obtained by absorbing the group agglutinins in an antiserum for a particular organism by treatment with an emulsion of another bacillus of the same group. The group serum was simply a serum prepared against another bacillus of the group, provided that it was rich in group agglutinins. When agar plates were prepared and individual colonies were separately tested, it was found that the bacilli of some colonies were agglutinated by the specific serum, but practically not at all by the group serum; while the converse held in the case of other colonies. In the mass culture there are accordingly two kinds of colony different in their antigenic structure, one containing the specific antigen, the other containing a group antigen. When, however, subcultures are made from a colony of either kind, the other soon reappears; that is, the specific or group character is not entirely stable. Among the motile forms of these organisms both flagellar (H) and somatic (O) antigens determine serological characters, and it has been shown that the diphasic character described above depends on the H antigen. Thus, in one phase this antigen is highly specific, in another it exhibits well-marked group characters.

Apart from the H antigenic components common to different types in the group phase, there may also be common O constituents among certain organisms, and even in the specific phase different types may possess similar H antigenic factors. Further, the whole group can be arranged in subgroups according to similarities in

their O antigens. These relationships are illustrated in the Table which follows on page 544.

The occurrence of rough variants among these organisms introduces a further difficulty in their serological investigation, since the "rough" transformation is associated with a change in the O antigen and a loss of its specific characters.

The Table shows the antigenic factors of several representative types, and illustrates the inter-relationships dependent on the existence of factors common to two or more different organisms of the group.

It will be seen that these organisms fall broadly into seven subgroups according to their O antigens: (1) Antigen I and II or III, e.g. *B. paratyphosus A*; (2) Antigens IV or IV and V, e.g. *B. paratyphosus B* and *B. aertrycke*; (3) Antigens VI and VII or VIII, e.g. *B. paratyphosus C* and *B. suipestifer*; (4) Antigen IX, e.g. *B. typhosus*, *B. enteritidis* and *B. pullorum*; (5) Antigens X and III; (6) Antigen XI; (7) Antigen XIII. Some occur only in the specific phase, e g. *B paratyphosus A*; some have been found only in the non-specific phase, e.g. *B. suipestifer* Kunzendorf type; many are diphasic, e.g. *B. paratyphosus B*. In the non-specific phase these organisms show marked similarities in their antigenic structure. Certain types are normally non-motile and lack H antigens, e.g. *B. pullorum*.

B. paratyphosus A is quite distinct antigenically from the others with the exception of the Senftenberg and Sendai types

The *Senftenberg* type has been recorded in cases of food-poisoning. It is related to *B. paratyphosus A* through its O antigen, and to *B. enteritidis* and Derby type through one H constituent. The Newcastle variety differs only in its inability to produce H_2S.

B. paratyphosus B shows close relationships to various other types, e g. *B. aertrycke*, in its O antigens; it is diphasic but in the specific phase its H antigen is highly distinctive.

B. aertrycke, like *B. paratyphosus B*, has marked group relationships through its O antigen; it is diphasic; in the specific phase it is related to the Aberdeen type which belongs to a different subgroup

The *Stanley*, *Reading* and *Derby* types have been reported in cases of food-poisoning. The last-named has also been found in pigs. Through their O antigens they are related to *B. paratyphosus B*.

B. abortus-equi has been found in equine abortion. It shares its O constituent with *B. paratyphosus B*. It occurs only in the specific phase and has H components in common with certain other types.

B. abortus-ovis, described in abortion of sheep, is diphasic and has the same specific H antigen as *B. suipestifer*. Through its O antigen it is also related to *B. paratyphosus B*.

B. paratyphosus C and *B. suipestifer*.—The former, also known as the Hirschfeld type, has been observed in cases of enteric fever in Eastern Europe and British Guiana. It shows little antigenic difference from *B. suipestifer* though distinguishable by biochemical reactions. Reference has been made earlier to the occurrence of the latter organism in pigs and its occasional association with enteritis in man. The *Kunzendorf* type differs from *B. suipestifer* in its monophasic character, occurring only in the non-specific

Type.	O Antigen.	H Antigen. Specific Phase.	H Antigen. Non-specific Phase.
paratyphosus A	I, II	a	—
Senftenberg var. Newcastle	I, III	g s	—
paratyphosus B	IV, V	b	1, 2
aertrycke (*S. typhi-murium*)	IV, V	i	1, 2, 3
Stanley	IV, V	d	1, 2
Reading	IV	e h	1, 4, 5
Derby	IV	f g	—
abortus-equi	IV	e n x	—
abortus-ovis	IV	c	1, 4, 6
paratyphosus C (Hirschfeld)	VI, VII	c	1, 4, 5
suipestifer (*S. choleræ-suis*)	VI, VII	c	1, 3, 4, 5
suipestifer (monophasic) (*S. choleræ-suis* var. Kunzendorf)	VI, VII	—	1, 3, 4, 5
Thompson	VI, VII	k	1, 3, 4, 5
Thompson var. Berlin	VI, VII	—	1, 3, 4, 5
Oranienburg	VI, VII	m t	—
Potsdam [1]	VI, VII	e n l v	—
Bareilly	VI, VII	y	1, 3, 4, 5
Newport	VI, VIII	e h	1, 2, 3
morbificans bovis	VI, VIII	r	1, 3, 4, 5
typhosus	IX	d	—
enteritidis	IX	g o m	—
enteritidis var. Dublin	IX	g p	—
Sendai	IX	a	1, 4, 5
Eastbourne	IX	e h	1, 3, 4, 5
gallinarum	IX	(non-motile)	1
pullorum	IX	(non-motile)	1
Dar-es-salaam	IX	e n l w	—
London	X, III	l v	1, 4, 6
anatum	X, III	e h	1, 4, 6
Aberdeen	XI	i	1, 2, 3
Poona	XIII	z_1	1, 4, 6

Based on Classification of Salmonella Subcommittee of the International Society of Microbiology, see *Journal of Hygiene*, 1934, vol. xxxiv, 333.

In this classification different O antigenic components are represented by Roman numerals, specific H components by letters, and non-specific H components by Arabic numerals.

For information regarding other types the paper cited should be consulted.

[1] Kauffmann has recently shown that certain types formerly regarded as monophasic are in fact diphasic: thus the Potsdam type (with H antigens *e*, *n*, *l*, *v*), in one phase possesses antigens *e* and *n* and in the other *l* and *v*. He indicates that the terms "specific" and "group" may come to be inapplicable to the respective antigens of the diphasic types.

phase. The organism has been described in both enteric fever and enteritis in man.

The *Thompson*, *Oranienburg*, and *Potsdam* types have been isolated from food-poisoning. Though related to *B. suipestifer* they have different specific H antigens. The *Berlin* variety of the *Thompson* type occurs only in the group phase.

The *Bareilly* type was described in enteric fever in India. While its O components are the same as those of *B. suipestifer*, it has a distinctive H antigen in the specific phase.

Newport type.—This organism has been found in food-poisoning. It is related to *B. suipestifer* but has the same specific H antigen as the *Reading* and *Eastbourne* types.

B. morbificans bovis has been isolated from an infected bovine and from food-poisoning in man. It also has an O relationship to *B. suipestifer*.

B. typhosus is a monophasic type. Its O antigen is the same as that of *B. enteritidis* and several other types. Its specific H antigen is identical with that of the *Stanley* type.

B. enteritidis and the *Dublin* type have the same O antigen as *B. typhosus* and like it occur only in the specific phase. Their H antigens are different from one another (though they have one common factor) and from the typhoid bacillus. Reference has been made above (p. 540) to the occurrence of *B. enteritidis* in animals. The *Dublin* type is a frequent pathogen in bovines and has been isolated also from other domestic animals. It has been reported in human cases of enteric fever.

The *Sendai* type though possessing the same O antigen as *B. typhosus* shares with *B. paratyphosus A* a common component in the specific phase. As mentioned earlier it has been isolated from enteric fever in Japan.

Eastbourne type.—This organism was described in a case of enteric fever. It is related through its O antigen to *B. typhosus*, but has the same H antigens (in the specific phase) as the *Reading* and *Newport* types.

Bacillus pullorum.—This organism produces a disease of chicks known as "bacillary white diarrhœa." It can be isolated from the fæces, internal organs, and blood, and the infection is transmitted through the egg from the parent bird. Adults have been shown to carry the organisms without obvious signs of infection, though such carriers can be recognised by agglutination reactions with the serum and a culture of *Bacillus pullorum*. The organism corresponds in general characters to the typhoid-paratyphoid group, but is non-motile. It ferments, usually with gas-production, glucose, mannitol, arabinose, and rhamnose, but has no action on lactose, dulcitol, saccharose, maltose, and inosite. Indole is not formed. This organism possesses the same somatic antigen as the typhoid bacillus.

Bacillus gallinarum occurs in a disease of fowls sometimes spoken of as "fowl typhoid." It resembles *B. pullorum* and is non-motile, but is non-gas-producing. It ferments glucose, mannitol, arabinose, maltose, and sometimes dulcitol. It has the same antigen as *B. pullorum* and cannot be distinguished from it by serum reactions.

The *Dar-es-salaam* type was isolated from a febrile illness in the human subject. It belongs to the same subgroup as *B. typhosus*

and has H components in common with various other types. It is stated to liquefy gelatin slowly.

London type and *Bact. anatum*.—These organisms are similar in their O antigens but differ in their specific H components. The former was isolated from human fæces; the latter was reported in an epizootic of ducklings.

The *Aberdeen* and *Poona* types each represent separate subgroups and have distinctive O antigens. The *Aberdeen* type in the specific phase has the same H antigen as *B. aertrycke*. It was isolated from a case of gastro-enteritis. The *Poona* type was also reported in gastro-enteritis. Its H antigen in the specific phase is quite distinctive among the various H components of the group.

Serological Identification of Salmonella Types

In the first place the determination of the O antigen affords a means of assigning an organism to its particular subgroup (see Table). For this purpose O-agglutinating antisera for representatives of these subgroups are required, e.g. *B. paratyphosus A*; *B. paratyphosus B*; *B. suipestifer* (monophasic) and Newport type; *B. enteritidis*, etc. A direct agglutination reaction with one of these would point to the type of O antigen of the unknown organism, and the result can be confirmed by absorption tests In the next place, in case the unknown organism be diphasic, the phase has to be ascertained. For this purpose an H-agglutinating antiserum for the monophasic *B. suipestifer* (Kunzendorf)—which exists only in the group phase—is tested with a formolised suspension of the unknown strain; if large flake agglutination results the organism is probably in the group phase and from it a specific-phase derivative must be separated. This can often be secured by plating, so as to obtain separate colonies. A loopful from each of a number of colonies is emulsified on a slide in a drop of antiserum (in low dilution) to the *B. suipestifer* (monophasic) referred to above. The presence or absence of agglutination is then noted —the object being to identify colonies which do not agglutinate with this serum and which therefore are presumably in the specific phase. Colonies of this type are subcultured and formolised suspensions of these subcultures are tested for agglutination with pure specific-phase H antisera for the various types in the particular subgroup to which the organism has already been assigned. The result is confirmed by absorption tests. In dealing with a monophasic type which occurs only in the group phase, the H antigens can be identified by a similar method. A non-motile variant can only be identified serologically as regards its O antigen. (For fuller information the original papers on this subject should be consulted.)

Dysentery Bacilli

Dysentery—a clinical term applied to cases characterised by tenesmus and the frequent passing of stools containing blood and mucus—has long been recognised as including several etiologically distinct conditions. There are two main types of

dysentery—*amœbic* due to the *Entamœba histolytica* (Chapter XXVI); and *bacillary*, due to *B. dysenteriæ*, a group of organisms comprising a number of different types. These are related biologically to the coli-typhoid group. It should be noted that cases with the clinical features of food poisoning may sometimes be due to infection with dysentery bacilli.

The first organism of this type to be described is that now known as *B. dysenteriæ Shiga*, observed by Shiga (1898) in cases of bacillary dysentery in Japan. Subsequently workers in different parts of the world confirmed Shiga's observations (Kruse and others), and similar organisms differing from the Shiga type in certain biological characters were also described (Flexner, Hiss and Russell, and others). It was further recognised that epidemics of dysentery occurring from time to time in mental hospitals were due to this group, and that the same organisms were responsible for cases of acute enteritis in infants and children—the so-called "summer diarrhœa." Important additions were made to our knowledge of bacillary dysentery during the war, when this condition assumed serious proportions among troops, especially in those campaigns carried on in tropical and sub-tropical areas. The evidence for the relationship of these organisms to the disease consists chiefly in their constant presence in the dejecta in the early stage of the illness and in the agglutination of the associated strains by the serum of patients, but confirmatory evidence has also come from animal experiments and from the therapeutic effects of specific antisera. While different biological types of *B. dysenteriæ* are recognised, they all present certain common characters and constitute a fairly well-defined group. The differentiation of types depends on differences in fermentation and biochemical reactions and in serological characters.

The following are the characters common to the classical or typical members of the group:

Morphological Characters.—The bacilli morphologically resemble the typhoid bacillus. They sometimes show a tendency to the cocco-bacillary form. No spore formation occurs. The organisms are non-motile; they are stained readily by the ordinary dyes, and are Gram-negative.

Cultural Characters.—On plates of nutrient agar the colonies resemble those of the typhoid bacillus, being usually of smaller size and less opaque than those of the *B. coli*. "Rough" colony variants may be observed in laboratory cultures, and sometimes a variant characterised by "mucoid" colonies (Fletcher). In *gelatin* stab culture no liquefaction results. On

MacConkey's medium the colonies are pale and colourless. In *broth* a uniform turbidity is produced. In *litmus milk* there is developed at first a slight degree of acidity, which is followed, in certain types of dysentery bacilli, by a phase of increased alkalinity; no coagulation of the milk occurs. On *potato* the organism forms a transparent or whitish layer, which, however, in the course of a few days assumes a dirty grey colour, with some discoloration of the potato at the margin of the growth.

As has been indicated, different types behave differently in biochemical and serological reactions. They all ferment glucose without gas production, and with the exception of certain atypical varieties (referred to later) do not ferment lactose. In all fermentation reactions they are non-gas-producing (*vide* Table, p. 557). The classical Shiga type (*Shigella dysenteriæ*) has no action on saccharose, mannitol, maltose, or dulcitol, and does not produce indole in peptone water. Another type (*Shigella paradysenteriæ*), originally described by Flexner, differs from the Shiga variety in the fermentation of mannitol and maltose, and in the production of indole. Hiss and Russell described a further type which is similar to the Flexner variety, but differs in the absence of maltose fermentation, and designated it the "Y" type. Thus the typical dysentery bacilli have been differentiated into two main sub-groups—the "non-mannitol-fermenters" (Shiga) and the "mannitol-fermenters" (Flexner-Y), which latter do not form a homogeneous group either in biochemical reactions or in serological characters. The mannitol-fermenting strains vary in the production of indole and the fermentation of maltose. Indole production even in the same strain may be variable. As a further criterion of identity the former react specifically with an agglutinating serum for a known Shiga strain, while the latter are generally agglutinated by an antiserum to the classical Y strain (*vide infra*) or alternatively by a polyvalent antiserum to the Flexner-Y sub-group.

It has been shown that the organisms of the Flexner-Y type possess four different antigenic components designated V, W, X, Z, one of which may predominate in a particular strain so as to give it a separate serological stamp; on this basis Andrewes and Inman classified the group into five main races, namely, V, W, X, Y, and Z, their Y race corresponding generally to the classical Y type. Two sub-races VZ and WX were also described. They regarded the Y race as presenting a mixture of the V, W, X, and Z components more evenly balanced

than in the other races. Thus an anti-Y serum has a wider agglutination range than sera from other strains.

According to Boyd, from a study of these organisms in India, in addition to Andrewes' recognised races of the Flexner subgroups, strains are encountered embodying considerable variation of antigenic complex. He has also found that variation in antigenic structure occurs among different colonies from the same culture of a dysentery bacillus of this subgroup.

Relation to the Disease.—The organism has been found in large numbers in the dejecta, especially in the early stage of the illness, where it may be present in almost pure culture. It does not appear to spread deeply or to invade the general circulation. In the later stages it may be relatively scanty in the stools and difficult to isolate. Apparently the organisms become progressively less numerous during the illness, and may ultimately disappear from the stools, even while the condition is still active. As they diminish, certain other types of intestinal bacteria appear in considerable numbers in the stool, *e.g.* Morgan's bacillus and allied organisms, paracolon bacilli (*vide* Table, p. 556), *B. fæcalis alkaligenes*, *B. proteus*, fæcal streptococci, etc. When complete recovery occurs, these give place to the usual coliform flora. MacConkey's lactose agar medium is specially suitable for isolation from stools. As the typical forms of *B. dysenteriæ* are non-lactose-fermenters, the colourless colonies which develop after twenty-four hours are picked out for further investigation.

In the severe and acute cases where death may occur in from one to six days, the chief changes are a marked swelling and corrugation of the mucous membrane of the colon, with hæmorrhage and pseudo-membrane at places. There is extensive coagulation-necrosis with fibrinous exudation and abundance of polymorphonuclear leucocytes, and the structure of the mucous membrane, as well as that of the muscularis mucosæ, is often lost in the exudation. Sometimes ulceration occurs; there is also great thickening of the submucosa, with infiltration of leucocytes, these being chiefly of the character of plasma cells. In the more chronic forms the changes correspond, but are more of a proliferative character. The mucous membrane is granular, and superficial areas are devoid of epithelium, while ulceration and pseudo-membrane are present in varying degree. In the stools the presence of a large number of markedly degenerated polymorphonuclear leucocytes, macrophages and red corpuscles, with the absence of *Entamœba histolytica*, points to bacillary dysentery; and in this way a tentative diagnosis may

be made by simple microscopic examination of the stool pending the fuller bacteriological investigation of the case.

Agglutination Reactions.—The serum of patients may agglutinate the particular causal type of dysentery bacillus, and the reaction has been applied in diagnosis. The reaction is often well marked after from six to seven days in the acute cases. In many cases, however, even after a longer interval the serum may fail to react. Agglutination of the Shiga bacillus in a serum dilution of 1 : 50 is usually accepted as being of diagnostic significance. The case of the Flexner-Y type is more difficult; it is susceptible to agglutination by normal sera to such an extent that probably a positive result with lower dilutions than 1 : 100 cannot be taken as indicating the presence of infection. The serological heterogeneity of the Flexner-Y types (*vide supra*) renders the practical application of the agglutination test difficult for diagnostic purposes, and in routine laboratory diagnosis the isolation of the causative organism constitutes the most conclusive method of bacteriological diagnosis. The application of agglutination tests with immune sera for the identification of strains has been referred to above.

Pathogenic Properties.—Generally it is impossible to produce any effect on laboratory animals by infection *per os*. Shiga, however, obtained characteristic effects by introducing the organism into the stomach of young cats and dogs, and confirmatory results were obtained by Flexner. Such attempts have been specially successful when the virulence of the organism has been previously exalted by intraperitoneal *passage*. The dysentery bacilli if recently isolated exhibit marked pathogenic effects when introduced intravenously in rabbits, a minute amount of culture being sufficient to produce a lethal result. The organisms show marked enterotropism, and inflammatory changes occur in the mucosa of the small and large intestine with frequently excessive hæmorrhages. The bacilli can be recovered from the contents of the intestine, where they may be present in large numbers. These results may also be produced by subcutaneous injection. In certain cases, apparently well authenticated, a dysenteric condition has followed in the human subject from ingestion of pure cultures of the organism.

Toxins.—It has now been well established that the Shiga type of dysentery bacillus produces a toxin (p. 190) which can be separated from fluid cultures by filtration. While this toxin resembles the exotoxins in its specialised action and its ability to incite the formation of a specific antitoxin, its presence in

culture-filtrates is due to autolysis of the bacterial cells following their death in the culture (Okell and Blake). The results of most observers show that the Flexner-Y strains do not produce a similar toxin. In this connection it is noteworthy that in general the Shiga strains, as contrasted with the other varieties, are associated with the most severe clinical form of the disease. The toxin of the Shiga type is extremely active in animals, especially rabbits, and, however introduced into the body, may produce a hæmorrhagic enteritis with often a pseudomembranous exudate on the surface of the mucosa. According to Kanai this toxin in rabbits affects also the central nervous system (medulla and spinal cord), with resulting paralysis which is a frequent result of inoculation. The toxin is fairly resistant to heat, withstanding temperatures up to 70° C. without loss of its properties.

An aggressin effect (*vide* p. 192) has also been described in the case of the dysentery bacillus.

Other Types of Dysentery Bacilli.—During the war, bacillary dysentery was extensively investigated, and while the classical types of bacilli formed a large proportion of the strains isolated from cases, "atypical" varieties were constantly met with. These were a prominent feature of dysentery in the Near East and were found in large numbers in the stools in early cases. Some of them corresponded in all their cultural and biochemical reactions to the Flexner-Y types, but failed to react to specific agglutinating sera for these organisms. Certain strains when repeatedly subcultured apparently underwent spontaneous changes in their biochemical characters and displayed fermentative reactions, *e.g.* fermentation of saccharose, dulcitol, lactose, which clearly differentiated them from the classical types. Other strains on first investigation showed certain biochemical differences from the classical types, and also reacted negatively with agglutinating antisera. All these types, however, presented characters in common with the recognised dysentery bacilli—being non-motile Gram-negative bacilli, non-gelatin-liquefying, non-gas-producing, fermenting glucose, but varying in the fermentation of saccharose, mannitol, dulcitol, and lactose, and the production of indole. Lactose-fermenting strains otherwise similar in biochemical characters to the Flexner type were observed. The dysentery bacilli now designated *Schmitz type*, *B. alkalescens* and *B. dispar* (*vide infra*) correspond to certain biological types reported by Mackie in the Near East during the war. The various types met with were proved when recently isolated to be extremely virulent on intravenous injection of rabbits, producing a characteristic hæmorrhagic enteritis similar to that resulting from inoculation with the classical strains. As regards the illness due to these organisms, the majority of cases were of the milder type, though severe conditions were not infrequently met with in which atypical organisms were present in large numbers in the early stage.

In recent years further attention has been paid to certain types of dysentery bacilli which differ from the classical forms and some of these have been found responsible for a proportion of cases of dysentery in various parts of the world :

Sonne Type.—A group of organisms biologically and serologically distinct from the Flexner-Y types was recognised by Thjøtta and Sonne as dysentery-producing organisms in Norway, Sweden, and Denmark. These organisms are similar to Flexner-Y strains and ferment mannitol, but late fermentation of saccharose and lactose occurs. Indole is not usually formed. Slow acid formation with clotting may occur in milk. It should be noted that the colonies of this organism when they first appear on MacConkey's medium are colourless though they may redden later. Cases and outbreaks due to this type have been described by various observers in Great Britain. Organisms of the Sonne type form a homogeneous group and can be identified by their serological reactions with a specific antiserum

Schmitz Type.—This organism is similar to *B. dysenteriæ* (Shiga), differing, however, in the formation of indole. It has been described in dysentery cases and outbreaks by various observers.

Bacillus alkalescens—This type resembles the Flexner-Y subgroup but ferments dulcitol. It alkalinises milk and produces indole. The evidence available indicates that this organism may be responsible for a dysenteric condition.

Bacillus dispar differs from the Flexner-Y subgroup in its fermentation of lactose and saccharose, these sugars, however, being fermented slowly. It occasionally ferments dulcitol. Indole is formed, and milk is slowly acidified and clotted. It can be differentiated from the Sonne type by its positive methyl-red reaction. Strains are serologically heterogeneous. Some observers have questioned the pathogenicity of this organism. It seems probable, however, that *B. dispar* is etiologically associated with dysenteric conditions which are generally less severe than those due to the typical dysentery bacilli.

Gettings Type.—This organism resembles the Flexner-Y subgroup in biochemical characters and produces indole but is serologically distinct from Flexner-Y strains.

Boyd has described strains isolated from dysentery in India, which exhibit the biochemical reactions of the Flexner-Y subgroup though different in antigenic structure from any of these organisms. He observed, however, dissociation of such strains and was able to derive from them a variant corresponding serologically to the Flexner-Y subgroup. It seems possible that the serological definition of the Flexner-Y subgroup according to the antigenic structure described by Andrewes and Inman is too restricted.

"*Newcastle dysentery bacillus*"—This organism has been reported in outbreaks of a dysentery-like illness by Clayton and Warren, and others. It resembles the dysentery group only to a limited extent. It has the general characters of the coli-typhoid organisms. Glucose, dulcitol and maltose are fermented, but not, as a rule, lactose, saccharose, or mannitol. When grown in sugar media prepared with peptone water as the basis, gas production is slight or inapparent ; when lemco broth is used, however, gas production is distinct. The strains of this type are serologically similar and distinct from the recognised dysentery bacilli.

Immunisation.—Both large and small animals have been immunised against the dysentery bacilli and also against toxic filtrates. In the former case the immunisation has been commenced either with non-lethal doses of living cultures, or with cultures killed by heat. The nature of the immunisation is probably complex. When cultures have been used, a bactericidal serum is developed in which immune-bodies and complement (*vide* Chapter VI.) are concerned. When toxin alone is used for immunisation, an antitoxic serum is produced. According to some results, animals immunised with cultures are immune against the toxin, and *vice versa.*

Antisera prepared by immunising large animals against the dysentery bacilli have now been extensively applied in the treatment of acute cases. Polyvalent sera have generally been used. Shiga originally reported favourably regarding the therapeutic value of a polyvalent serum used in Japan in large numbers of cases. The active principles of such sera are both antitoxic and antibacterial. While reports as to their practical value have varied considerably, it may be said that in the severe cases, if sufficiently large doses are given (*e.g.* 50–100 c.c.) and repeated if necessary, favourable results are obtained. In the most acute cases the serum should be given intravenously.

Prophylactic Vaccination.—A difficulty attending the application of vaccines is the extremely toxic effects exerted by killed cultures when injected subcutaneously even in relatively small doses. Shiga obviated this by using a sensitised vaccine (*vide* p. 206), and this method has been followed by others. Oral administration of dysentery vaccines according to Besredka's method has also been applied and successful results claimed. Vaccine prophylaxis has not, however, been so extensively applied as to allow of conclusions being drawn regarding its practical value.

Bacteriophage Therapy.—D'Herelle has advocated the use of a dysentery bacteriophage in the treatment of the disease, and others have reported favourably on the results of this form of therapy. Its value, however, is doubtful (Fletcher and Kanagarayer; Riding).

Bacilli of Infantile Diarrhœa

The etiology of this condition, so prevalent in infants and children in the warmer seasons of the year, has attracted considerable attention from the bacteriological standpoint. It is doubtful whether the condition is to be regarded as due to a

single specific agent. Certain of the more severe cases occurring in this country at the present time and in other temperate climates are found on investigation to be due to the dysentery bacilli, mostly of the Flexner-Y type. Recently the Sonne type has also been noted. Such cases occur both sporadically and in groups, and while the stools may present the typical appearances of dysenteric dejecta with blood and mucus present, such appearances may be absent, and the condition would not be designated dysentery in the clincial application of the term.

A careful investigation of the disease in Britain was made some years ago by Morgan, who found no evidence of the association of dysentery bacilli with the condition. In 63 per cent. of the cases investigated, however, he isolated from the stools and intestine a type of organism now generally designated "Morgan's No. 1 bacillus" (*Salmonella morgani*), which is a motile Gram-negative bacillus belonging to the coli-typhoid group and possessing characteristic fermentative reactions. It produces acid and gas in glucose, but has no effect on lactose, saccharose, mannitol, maltose or dulcitol; it produces indole; gelatin is not liquefied. It causes diarrhœa and death in young rabbits, rats, and monkeys, when these animals are fed on cultures. Morgan also found that in diarrhœa cases, the lactose-fermenters, so characteristic of normal fæces, are relatively less numerous and tend to be replaced by non-lactose-fermenting types.

In a study of simple diarrhœal conditions, apart from clinical dysentery, occurring in troops during the Mediterranean campaign, various non-lactose-fermenting Gram-negative bacilli, *e.g.* Morgan's No. 1 bacillus and similar types, paracolon bacilli (*vide* Table, p. 556), *B. fæcalis alkaligenes*, *B. proteus*, etc., were met with in stools and were present often in very large numbers, almost replacing the usual coliform bacilli (Mackie). These organisms were also met with as concomitants of the dysentery bacilli (*vide supra*) in typical dysenteric cases. The question remains unsettled as to whether such organisms are pathogenic types or whether they are simply intestinal commensals normally scanty, but under certain conditions capable of replacing the usual coliform flora. The evidence collected on this subject is highly suggestive, however, that certain of these organisms are pathogenic entities responsible for diarrhœal conditions not only in young subjects, but also in adults.

Bacillus Fæcalis Alkaligenes (*Alcaligenes fæcalis*).—This organism may conveniently be considered here. It is a motile,

Gram-negative, non-sporing bacillus resembling the typhoid bacillus in its morphological and general cultural characters. It is, however, devoid of fermentative properties towards any of the carbohydrate substances used in the testing of the organisms of the coli-typhoid group (*vide* Table, p. 556). It does not liquefy gelatin. The classical type is actively motile, but reports as to the number and arrangement of its flagella vary. Multiple peritrichous flagella have been described, but strains are met with possessing a single terminal flagellum like that of certain vibrios. Non-motile strains with otherwise the same characters as *B. fæcalis alkaligenes* may be met with, and it seems probable that organisms classified under this designation represent a number of varieties.

This type of organism may occur occasionally in small numbers in fæces from healthy persons. In certain pathological conditions of the bowel large numbers may be found in the stool, often replacing to some extent the normal *B. coli* flora, *e.g.* in late cases of bacillary dysentery (*q.v.*) and in non-dysenteric diarrhœa. Blood infections with *B. fæcalis alkaligenes* have also been described in cases presenting a transient febrile illness.

Isolation and Differentiation of Coli-Typhoid Bacilli

The existence of a group of intestinal diseases with similar clinical features caused by closely allied bacteria makes the differentiation of these affections and of the causal bacteria a somewhat difficult problem. The difficulty is increased where, as in the conditions existing during the late war, several of these diseases may be simultaneously prevalent, each on a considerable scale. The best solution of the bacteriological problem is found in the isolation of the organisms from the blood or stools of the patient, but here often the best methods may fail to yield cultures, especially when, as has often been the case, the individual does not come under observation till the acute phase of the disease has passed. Moreover, cases occur where more than one pathogenic organism may be present. The isolation method should, however, invariably be attempted.

Post mortem, organisms may be isolated from the intestine, particularly from ulcerative lesions if such occur, as in enteric fever and in dysentery; cultures may be obtained from the gall-bladder, and here the causative organism may occur in a state of purity as in enteric fever; the particular organism may also be isolated from the spleen, mesenteric glands, and even the heart blood, as in enteric fever and in bacterial food-poisoning.

During life, the bacilli may be obtained in culture in the following ways:

(*a*) *From the Blood.*—The bacilli of the group may be isolated from the blood by ordinary methods (see p. 165), but a special

TABLE SHOWING CHARACTERS OF THE MORE IMPORTANT REPRESENTATIVES OF THE COLI-TYPHOID GROUP

	Motility. 1	Gelatin. 2	Glucose. 3	Lactose. 4	Dulcitol. 5	Saccharose. 6	Mannitol. 7	Adonitol. 8	Inulin. 9	Inosite. 10	Xylose. 11	Arabinose. 12	Rhamnose. 13	Milk. 14	Indole. 15	Voges and Proskauer Reaction. 16	Production of H_2S.[4] 17	Methyl-red reaction. 18	Utilisation of Sodium citrate. 19
B. coli communis (Escherich)	+	-	A.G	A.G.	A.G	-		-	-	-				A.C.	+	-		+	-
B. MacConkey No. 71 ("B. coli communior") . .	+	-	A.G.	A.G.	A.G.	A.G.	A.G.	-	-	-				A.C.	+	-		+	-
B. lactis aerogenes (atypical B. coli)	-	-	A.G.	A G.	-	A.G.		A G.	-	A.G.				A.C.	-	+		-	+
Pneumobacillus type . .	-	-	A.G.	A.G. or A.	A.G.	A.G.		A G.	-	A.G.				A.C.	-	-		+	+
B. coli anaerogenes type .	+	-	A.	A.	A.	-	A.	-	-	-				A.	+	-		+	-
B. cloacæ type . . .	+	+	A.G.	A.G.	-	A.G.	A.G.	A.G	-	A.G.				A.C.	-	+		-	+
B. "paracolon" types [1] .	+ or -	-	A.G.	-	- or A.G	-	A.G.								+ or -				
B. Morgan No. 1 . . .	+	-	A.G.	-	-	-	-								+				
B. typhosus	+	-	A	-	- or A (late)	-	A			-	A.	— or A (late)	-	Pract. no change	-		+		
B. paratyphosus A. . .	+	-	A.G	-	A.G.	-	A.G.			-	-	A.G. — or A (late)	A.G.	ditto.	-		-		

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
B. paratyphosus B.	+	-	A.G.	-	A.G.	-	A.G.			A.G. (usually)	A.G.	A.G.	A.G.	Alk.	-		+		
B. enteritidis (Gaertner)	+	-	A.G.	-	A.G.	-	A.G.			-	A.G.	A.G	A.G	Alk.	-		+		
B. aertrycke	+	-	A.G.	-	A.G.	-	A.G.			A.G (usually)	A.G.	A.G.	A.G.	Alk.	-		+		
B. suipestifer[2]	+	-	A.G.	-	– or A. or A.G. late.	-	A.G.			-	A.G.	-	A.G.	ditto.	-		-		
B. paratyphosus C.—Hirschfeld type	+	-	A.G.	-	A.G.	-	A.G.			-	A.G.	A.G	A.G. (variable)	ditto.	-		+		
B. dysenteriæ (Shiga)	-	-	A.	-	-	-	-							Pract. no change	-				
B. dysenteriæ (Flexner-Y types)[3]	-	-	A.	-	-	-	A.							Pract. no change	+ or -				
B. dysenteriæ (Sonne)	-	-	A.	A. (late)	-	A. (late)	A							A.C. (late)	-				

+ in Motility column=presence of motility; in Gelatin=liquefaction; in Indole=formation of indole: in Voges and Proskauer=presence of reaction. —in Motility column=absence of motility; in Gelatin=no liquefaction; in Indole=absence of indole formation; in Voges and Proskauer=absence of reaction; in other columns=absence of change or reaction. "A."=Acid production; "G."=Gas; "C."=Clot; "Alk."=development of alkalinity.

[1] Mackie, *Journal of Hygiene*, 1919, xviii. 69.

[2] Monophasic type (Kunzendorf) produces H_2S.

[3] From *Maltose* acid (but no gas) may be formed or there may be no change.

[4] Tested by growing in nutrient agar containing 0·05 per cent. basic lead acetate; blackening of the medium indicates H_2S production.

Note.—In general, only those characters are given which are important in identification and differentiation.

Biochemical reactions of certain *Salmonella* types not specified in the Table may be summarised as follows:

B. morbificans bovis, Reading, Derby, Thompson, Bareilly, Potsdam, and London types—similar to *B. paratyphosus B.*;

B. abortus-equi, *Bact. anatum*, Senftenberg, Stanley, Newport, Oranienburg, Dar-es-salaam, Eastbourne, Aberdeen, and Poona types—similar to *B. enteritidis*.

B. abortus-ovis—similar to *B. enteritidis*, but does not ferment rhamnose and milk is practically unchanged.

Senftenberg var. Newcastle—differs from Senftenberg (*v. supra*) in absence of H_2S production.

method is often used. In this, 5 c.c. of the blood are placed in 10 c.c. sterilised ox bile, or in 50 c.c. sterilised 0·5 per cent. solution of sodium taurocholate; the mixture is incubated for one to seven days, and from time to time the presence of non-lactose fermenters is tested by inoculating MacConkey's medium. In typhoid and paratyphoid infections the organisms have been stated to have been isolated from the blood during the prefebrile stage and are very usually present during the first seven to ten days of the illness and in relapses. It may be said generally that the isolation of an organism of the group from the blood during an acute illness probably furnishes the most significant evidence as to its being the cause of the condition present. In bacillary dysentery a blood infection is not common.

(*b*) *From the Stools.*—In the case of a solid or semisolid stool, a dense emulsion is prepared in saline and allowed to stand in a tube till the solid particles have settled; one or two loopfuls from the supernatant fluid are then used for inoculating plates of MacConkey's medium by the methods described on page 88—the plate being incubated with the medium uppermost. The brilliant green and the tetrathionate enrichment methods may be recommended for the typhoid-paratyphoid group (p. 74). In any case the presence on MacConkey's medium of colourless colonies of Gram-negative bacilli constitutes presumptive evidence of the existence of pathogenic members of the coli-typhoid group in the fæces. Some of these colonies should now be picked off into peptone-water and into mannitol tubes. The former are used after a few hours' incubation for investigating motility—the latter for observing fermentation. Preliminary indications of the type of organism may be obtained as follows: growth in mannitol without acid or gas suggests the *B. dysenteriæ Shiga*, if the organism is non-motile, or Morgan's No. 1 bacillus, if motile; the development of acid without gas may be due to the *B. typhosus*, if the bacillus be motile, or to the *B. dysenteriæ* (*Flexner-Y*), if non-motile; a culture showing acid and gas associated with motility in the organism indicates one of the paratyphoid bacilli, *B. enteritidis*, etc. Agglutination observations may now be made (*vide infra*), and a set of fermentation and biochemical tests appropriate to the organism suspected to be present may be put up (*vide* Table, p. 556); it is well to include amongst these a gelatin tube, to exclude *B. proteus* types, and one of lactose—the latter to be kept under observation for some days in case the bacillus be a slowly lactose-fermenting *B. coli*. In this connection the fact must always be borne in mind, in dealing with any coli-typhoid bacillus, that its fermentative capacities may be only slowly manifested. A newly isolated organism may not show its full biochemical properties till it has been subcultured several times.

For satisfactory bacteriological examination of fæces it is essential that certain conditions should be observed with regard to the taking of specimens; these are detailed on p. 168.

In any extensive investigation of intestinal infections, atypical bacilli will from time to time be encountered which show variations from the characters of the classical type.

The fæces always constitute an important source of cultures in the diseases under consideration. In typhoid infections the organisms tend to be most numerous in the stools in the third week.

B. paratyphosus A is most numerous about the twelfth day and *B. paratyphosus B* at the end of the second week. It must be remembered, however, that compared with the number of *B. coli* in the stool the pathogenic organisms may be relatively scanty and technically difficult to isolate. For this reason selective enrichment methods have proved of great value in the isolation of the typhoid-paratyphoid group.

In bacillary dysentery, the organisms are usually very numerous during the first few days of the illness but gradually disappear from the stools and are replaced by various "concomitant" organisms (p. 549). Certain of these are non-lactose fermenters, *e.g.* B. Morgan No. 1, paracolon bacilli, etc.

(*c*) *From the Urine.*—In typhoid fever the bacilli are present in at least 25 per cent. of cases, especially late in the disease, probably where there are groups of the organisms in the kidney substance. The organism can also be found in paratyphoid infections. For methods of examining the urine, see pp. 169, 512. In these conditions bacilluria may occur in which the typhoid or paratyphoid bacilli are so numerous in the urine as to render it turbid.

The ultimate differentiation of the pathogenic members of the coli-typhoid group is effected by the study of their agglutination reactions with sera prepared by immunising animals with known typical strains of the particular organism. For identification, the suspected strain should agglutinate to approximately the "end-titre" of the serum, and tests must be carried out by the quantitative method (*vide* p. 138). Agglutinin-absorption tests may be required for the identification of the various *Salmonella* types, *e.g.* in cases of food poisoning (p. 141). Among these organisms the flagellar and somatic antigens may require to be separately identified and difficulties may be encountered owing to the diphasic character of certain types. The serological characters and identification of the specific pathogenic organisms of the coli-typhoid group have been dealt with in previous sections of this chapter.

In any extended investigation of intestinal infections, bacilli may be met with similar in biochemical and other cultural tests to specific pathogenic types though inagglutinable by antisera to the species they resemble. Certain of these are found on repeated subculture to agglutinate ultimately with the appropriate antisera and can thus be identified with a recognised type. A strain, therefore, which on first isolation conforms to a specific type but fails to react with the respective antiserum should be retested after several subcultures on artificial medium. Alternatively its identity may be established by the absorption test if it is found to absorb from an agglutinating serum for the particular type the agglutinins for this organism.

CHAPTER XVI

THE VIBRIO CHOLERÆ AND ALLIED ORGANISMS

Introductory.—In 1883 Koch discovered the organism called by him the " comma bacillus " and now generally known as the *Vibrio choleræ*. He obtained pure cultures of the organism from a large number of cases of cholera, and described their characters. The results of his researches were given at the first Cholera Conference at Berlin in 1884. This disease which is endemic in the region of the Ganges, caused repeated epidemics, with great mortality, in Europe in the nineteenth century.

In considering the bacteriology of cholera, it is to be borne in mind that the bacilli when ingested multiply quickly in the intestine and toxic effects tend to develop rapidly, *e.g.* as early as thirty-six hours. There occurs profuse watery discharge from the bowel —sometimes preceded by a " premonitary " attack of ordinary diarrhœa ; then there set in vomiting, shrivelling of the skin, cramps, suppression of urine and collapse. Loss of water and chlorides are largely responsible for these conditions, but it is likely that toxic products of the bacilli, in addition to acting locally on the intestine, are absorbed and affect specially the circulatory and thermo-regulatory mechanisms. This general toxic action is most evident in the serious symptoms at the stage of " reaction " in cases surviving the collapse ; but mixed infection may then be partly responsible. In rare cases, known as *cholera sicca*, general collapse occurs with remarkable suddenness, and is rapidly followed by a fatal result, while there is little or no evacuation from the bowel, through *post mortem* the intestine is distended with fluid contents. It is also to be noted that cholera is a disease of which the onset and course are much more rapid than is the case in most infective diseases, such as typhoid and diphtheria ; and further that recovery, when it takes place, does so more quickly.

Vibrio Cholerae (*Vibrio comma*).—*Microscopical Characters.*—The cholera vibrios, as found in the intestines in cholera, are small organisms measuring about 1·5 to 3 μ in length, and rather less than 0·5 μ in thickness. They are distinctly curved in one direction, hence the appearance of a comma (Fig. 116) ; most occur singly, but some are attached and curved in opposite directions, so that S-forms result. Longer forms are

rarely seen in the intestine, but in fluid cultures, they may grow into spiral filaments, showing a number of turns. Different strains may show considerable variation in their microscopic appearances. In film preparations made from the intestinal contents in typical cases, vibrios may be present in enormous numbers and in almost pure culture.

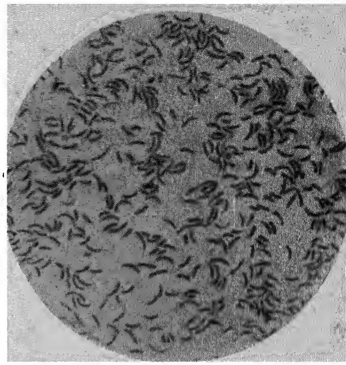


FIG. 116.—Cholera vibrios, from a culture on agar of twenty-four hours' growth. Stained with weak carbol-fuchsin. ×1000.

They possess very active motility, which is most marked in the single forms, and this is due to one terminal flagellum (Fig. 117) It is very delicate, and measures often two or three times the length of the organism. Cholera vibrios do not form spores. In old cultures the organisms may present great variety in size and shape. Some are irregularly twisted filaments, some globose, some clubbed at their extremities, and also showing irregular swellings along their course; others are short and thick, and may have the appearance of large cocci, often staining faintly. All these may be classed together as *involution forms* (Fig. 118), though some observers have regarded certain of the aberrant forms as phases in the life-history of the organism. It should be noted that the cholera vibrio after continued growth in culture medium becomes less typical in its appearance and the curvature is less obvious.

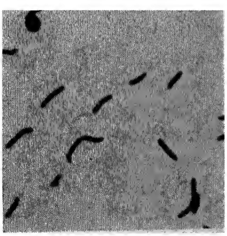


FIG. 117.—Cholera vibrios stained to show the terminal flagella. See also Plate IV., Fig. 20. ×1000.

Staining.—Cholera vibrios stain readily with the usual basic aniline stains, though crystal violet solution or weak carbol-fuchsin is specially suitable. They are Gram-negative.

Distribution within the Body.—The chief fact in this connection is that the vibrios are practically confined to the intestine. Certain observations show that they may be found sometimes

in the internal organs, and especially in the gall-bladder and biliary passages. Greig found in a large series of post-mortem examinations that the cholera organism was present in the gall-bladder in more than a quarter of the cases, and that in a considerable number of these distinct pathological changes were present. Another interesting fact observed by him was that in rabbits inoculated intravenously with the living organisms, infection of the gall-bladder and the formation of gall-stones not infrequently occurred. An important factor in the pathology of the disease, however, is the toxic effect on the bowel. In cases in which there is the characteristic "rice-water" fluid, the lower half of the small intestine is the part most affected. Its surface epithelium becomes shed in great part, and the flakes floating in the fluid consist chiefly of masses of epithelial cells and mucus, among which are numerous vibrios. The vibrios also penetrate the follicles of Lieberkühn, and may be seen lying between the basement membrane and the epithelial lining, which becomes loosened by their action. In some very acute cases there may be relatively little desquamation of epithelium, the intestinal contents being a comparatively clear fluid containing the vibrios in large numbers. In other cases of a subacute type, the intestine may show more extensive necrosis of the mucosa and a considerable amount of hæmorrhage into its substance, along with formation of false membrane at places. The intestinal contents in such cases are blood-stained and foul-smelling, there being a great proportion of other organisms present besides the cholera vibrios.

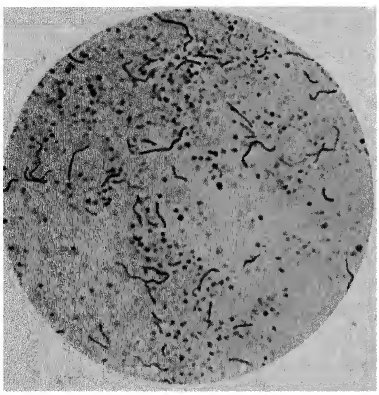


FIG. 118.—Cholera vibrios from an old agar culture, showing irregularities in size and shape, with numerous faintly-stained coccoid bodies—involution forms. Stained with fuchsin. ×1000.

Cultivation.—(For methods of isolation, see p. 576).

The cholera vibrio grows readily on all the ordinary media under aerobic conditions, and, except on potato, growth takes

place at the ordinary room temperature. The most suitable temperature, however, is 37° C., and growth usually stops about 16° C., though in some cases it has been obtained at a lower temperature. Abundant growth occurs on media with a sufficiently alkaline reaction to inhibit the growth of many intestinal bacteria, *e.g.* Dieudonné's medium, page 70. The optimum reaction is between pH 8·0 and 9·0.

On *agar,* the colonies after twenty-four hours' incubation are greyish-white transparent discs, corresponding in size to colonies of the coli-typhoid group, with a well-defined circular outline. A stroke culture gives a uniform growth of similar appearance. Older growths develop a brownish-yellow tint.

As in other bacterial groups, variation occurs as regrads the morphology of colonies, and "rough" variants may be observed which are analogous to the "rough" colonies of the coli-typhoid group. Colony variants have been studied by Balteanu, who has described three types: (1) "circumvallate rugose" colonies, which are small, yellowish, and opaque; (2) "ring" colonies, which often show an opaque centre and transparent border; (3) "opaque" white and adherent colonies, consisting of non-motile units and in serological tests containing only the O (somatic) type of antigen.

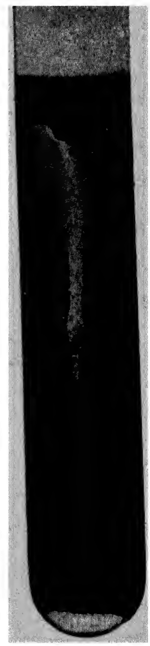


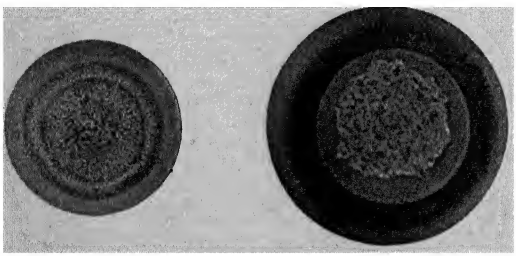
Fig. 119.—Stab culture of the cholera vibrio in gelatin—six days' growth. Natural size.

Gelatin.—On this medium the organism grows well and produces liquefaction. In stab cultivation at 22° C. a whitish line appears along the needle track, at the upper part of which liquefaction commences, and as evaporation quickly occurs, a small depression forms, which gives the appearance of an air-bubble. On the fourth or fifth day the following appearance may be seen. There is at the surface the depression described above; below this there is a funnel-shaped area of liquefaction, the fluid being only slightly turbid, but showing at its lower end thick masses of growth of a more or less spiral shape in the thin line of liquefaction (Fig. 119). (It is to be noted that considerable variations in the degree and rate of liquefaction of gelatin are observed.) At a later stage liquefaction

spreads and may reach the wall of the tube. When the organism is subcultured over a long period of time, it may lose to a large extent the property of liquefying gelatin.

On *gelatin plates* the colonies are somewhat characteristic. They appear as minute whitish points, visible in twenty-four to forty-eight hours, the surface of which, under a low power of the microscope, is irregularly granular or furrowed (Fig. 120, A, B); but considerable variations in the appearances are met with. Liquefaction occurs, and the colony sinks into the small cup formed, the plate then showing small sharply marked rings around the colonies.

Growing on *coagulated blood serum* the cholera vibrio slowly liquefies the medium. On *potato* with a sufficiently alkaline reaction and at a temperature of from 30° to 37° C. a moist



A B

FIG. 120.—Colonies of the cholera vibrio on a gelatin plate—three days' growth. A shows the granular surface, liquefaction just commencing; in B liquefaction is well marked.

layer appears, which assumes a colour varying from yellowish-brown to pink.

In alkaline *broth* the organism grows very readily, a general turbidity resulting in twelve hours at 37° C., while the surface shows a well-marked pellicle composed of vibrios in a very actively motile condition. Growth takes place under the same conditions equally rapidly in alkaline peptone solution (1 per cent. with 0·5 per cent. sodium chloride added).

Vibrio choleræ produces acid, without gas formation, from glucose, lævulose, galactose, saccharose, mannose, mannitol, maltose, starch, and dextrin; late fermentation of lactose, with acid production, may occur, namely, after two to three days, but different strains show variation in the power of fermenting this sugar.

In *milk* also the organism grows well, and produces no co-

agulation nor any change in its appearance, at least for several days.

On all the media the growth of the cholera vibrio is relatively rapid, and especially is this the case in peptone solution and in broth, a circumstance of importance in relation to its separation in cases of cholera (*vide* p. 576).

The cholera organism is one which grows much more rapidly in the presence of oxygen than in anaerobic conditions ; under strict anaerobiosis very little growth occurs.

Cholera-Red Reaction.—This is a fairly constant reaction, though it is not peculiar to the cholera vibrio. The test is made by adding a few drops of *pure* sulphuric acid to a culture in peptone solution (1 per cent.) which has been incubated for twenty-four hours at 37° C. ; in the case of the cholera vibrio a reddish-pink colour of nitroso-indole is produced. This is due to the fact that both indole and nitrite are formed. (A similar tube of uninoculated medium may be tested as a control.) Rough variants of *V. choleræ* and strains from carriers may fail to give the nitroso-indole reaction (Seal).

Hæmolytic Test.—The classical *V. choleræ* has usually been described as non-hæmolytic, whereas other vibrios likely to be confused with it are strongly hæmolytic when growing on blood agar, and produce well-marked areolæ of laking round the colonies. Strains of vibrios may be met with, serologically related to *V. choleræ*, which are definitely hæmolytic, *e.g.* the so-called El Tor vibrio (*vide* p. 573). Further, strains which appear to be non-hæmolytic after twenty-four hours' growth on blood agar may later produce a clear zone round each colony (Greig). It has, however, been found by several observers that the hæmolytic test is best carried out with a fluid culture. Greig recommends the addition of varying amounts, up to 1 c.c. of a three days' culture in alkaline broth to 1 c.c. of a 5 per cent. suspension of goat's corpuscles, the whole being made up to 2 c.c , and thoroughly mixed. The tubes are placed in the incubator for two hours at 37° C., and then in the ice-chest overnight, the results being read next day. He found after testing more than 300 strains of true cholera vibrios that none of them produced hæmolysis, whereas this resulted with organisms of the El Tor type referred to.

Reference has been made to the fact that strains of *V. choleræ* may produce clearing of blood agar though they are devoid of a true hæmolysin. Van Loghem first suggested that this effect is due to " hæmodigestion," and Kovacs has shown that the clearing effect also occurs when these organisms are growing on agar containing heated blood (*e.g.* " chocolate " agar, p. 69), whereas the El Tor vibrio does not clear this medium. The clearing of heated blood is possibly due to breaking down of hæmoglobin with the formation of alkaline hæmatin. Some vibrios produce both true hæmolysis and this clearing effect (on heated blood), though as shown above the two properties are often dissociated (Finkelstein).

Viability and Infection.—In its resistance to heat, the cholera vibrio corresponds with most spore-free organisms, and is killed in an hour by a temperature of 55° C., and much more rapidly at higher temperatures. It has comparatively high powers of resistance to cold, and has been found alive after being exposed for several hours to a temperature of −10° C. It is, however, killed by being kept in ice for a few days. Against the ordinary antiseptics it has comparatively low powers of resistance.

As regards the powers of resistance in ordinary conditions, the following facts may be stated. In cholera stools kept at the ordinary room temperature, the cholera organisms are rapidly outgrown by putrefactive bacteria, but in exceptional cases they have been found alive even after two or three months. In most experiments, however, attempts to cultivate them even after a much shorter time have failed. The general conclusion may be drawn from the work of various observers, that the vibrios do not multiply freely in ordinary sewage, although they may remain alive for a considerable period of time. Though we can state generally that the conditions favourable for the growth of the cholera vibrio are a warm temperature, moisture, a good supply of oxygen, and a considerable proportion of organic material, it is doubtful if it can flourish for any length of time as a saprophyte. The fact that the area in which cholera is an endemic disease is so restricted, tends to show that the conditions for growth of the vibrio outside the body are not usually supplied.

During epidemics the cholera organism has been cultivated from the stools of a considerable number of people suffering from slight intestinal disturbance, and even from the stools of quite healthy individuals; these may be regarded as "cholera carriers." Numerous observations, carried out both on convalescents and on contacts having the vibrio in the stools, show that in the great majority of cases it dies out after two or three weeks and usually earlier; it has, however, been found as long as twelve months afterwards. Greig has found that the excretion of the organism in the stools of carriers is of an intermittent character; accordingly several examinations are necessary before they can be pronounced free. As in the typhoid carrier, the organisms apparently persist in the gall-bladder. There is no doubt that carriers play an important part in the spread of the disease, and can originate epidemics.

Cholera organisms are, as a rule, rapidly killed by being

thoroughly dried, and thus they cannot be carried in the living condition for any great distance through the air, *e.g.*, in dust. Cholera is commonly transmitted by means of water or food contaminated by the organism, and there is no doubt that contamination of water supplies by choleraic discharges is the chief means by which communities are rapidly infected. It has been shown that if flies are fed on material containing cholera vibrios, the organisms may be found alive within their bodies twenty-four hours afterwards. And further, Haffkine found that sterilised milk might become contaminated with cholera organisms if kept in open jars to which flies had free access, in a locality infected by cholera. Thus infection may be carried also by this agency.

Experimental Inoculation.—In considering the effects of inoculation with the cholera organism, we are met with the difficulty that none of the lower animals, so far as is known, suffers from the disease under natural conditions. Accordingly, attempts to induce the multiplication of the organism within the intestine of animals, by artificially arranging favouring conditions, occupied a prominent place in the early experimental work. We shall give a short account of such experiments :

Nikati and Rietsch were the first to inject the organisms directly into the duodenum of dogs and rabbits, and they succeeded in producing, in a considerable proportion of the animals, a choleraic condition. These experiments were confirmed by other observers, including Koch. Thinking that probably the vibrio, when introduced by the mouth, is destroyed by the action of the hydrochloric acid of the gastric secretion, Koch neutralised this acidity by administering carbonate of soda to guinea-pigs, and some time afterwards introduced a pure culture into the stomach by means of a tube. As this method failed to give positive results, he tried the effect of artificially interfering with the intestinal peristalsis by injecting tincture of opium into the peritoneum, in addition to administering as before carbonate of soda. The result was remarkable, as thirty out of thirty-five treated animals died rapidly with symptoms of general prostration and collapse. *Post mortem* the small intestine was distended, its mucous membrane congested, and it contained a colourless fluid with small flocculi and the cholera organisms in practically pure culture. Koch, however, found that when the vibrios of Finkler and Prior, of Deneke (*vide infra*), and of Miller, were employed by the same method, a certain, though much smaller, proportion of the animals died from an intestinal infection. Though the changes in these cases were not so characteristic, they were sufficient to prevent the results obtained with the cholera organism from being used as a demonstration of the specific relation of the latter to the disease.

Some additional facts with regard to choleraic infection of animals may be mentioned. For example, Zabolotny found that in the marmot an intestinal infection readily takes place by simple feeding

with the organism, there resulting the usual intestinal changes, sometimes with hæmorrhagic peritonitis—the organisms, however, being present also in the blood. And of special interest is the fact, discovered by Metchnikoff, that in the case of young rabbits shortly after birth, a large proportion die of choleraic infection when the organisms are simply introduced along with the milk, as may be done by infecting the teats of the mother. Further, from these animals thus infected the disease may be transmitted to others by a natural mode of infection. In this infection of young rabbits many of the symptoms of cholera are present Many of these experiments were performed with the vibrio of Massowah, which is now admitted not to be identical with the classical cholera organism, others with a cholera vibrio obtained from the water of the Seine.

Experiments performed by direct inoculation also supply interesting facts.

Intraperitoneal injection in guinea-pigs is followed by general symptoms of illness, the most prominent being distension of the abdomen, subnormal temperature, and, ultimately, profound collapse. Of a young virulent culture one-tenth of a loopful will cause death in about twenty-four hours. There is peritoneal effusion, which may be comparatively clear, or may be somewhat turbid and contain flakes of lymph, according to the stage at which death takes place. If the dose is large, organisms are found in considerable numbers in the blood and also in the small intestine, but with smaller doses they are practically confined to the peritoneum. Kolle found that when the minimum lethal dose was used in guinea-pigs, the peritoneum might be free from living organisms at the time of death, the fatal result having taken place from an intoxication.

The cholera vibrio is markedly "enterotropic," and by the intravenous injection of cultures in rabbits, a pathological picture resembling that in human cholera can be produced (Mackie). The animals die in twenty-four to forty-eight hours, and before death there is marked diarrhœa; at autopsy the small intestine is markedly distended with a milky mucous fluid containing whitish flakes of desquamated epithelium and resembling the "rice-water" stool of cholera. Large numbers of vibrios are present in the intestine, and the gall-bladder is also heavily infected. It must be noted, however, that other vibrios produce a similar effect, *e.g.* the so-called paracholera vibrios.

It will be seen from the above account that the evidence obtained from experiments on animals is on the whole indicative of the specific pathological relationships of the organism, especially when it is borne in mind that animals do not in natural conditions suffer from the disease.

Experiments on the Human Subject.—Experiments have been performed in the human subject, and accidental infections have also occurred in laboratory workers. In the course of Koch's earlier work, one of the workers in his laboratory was seized with severe choleraic symptoms. The stools were found

to contain cholera vibrios in enormous numbers. Recovery, however, took place. In this case there was no other possible source of infection than the cultures with which the person had been working, as no cholera was present in Germany at the time. A considerable number of experiments have been performed, which certainly show that in some cases more or less severe choleraic symptoms may follow ingestion of pure cultures, whilst in others no effects may result. The former was the case, for example, with Emmerich and Pettenkofer, who made experiments on themselves, the former especially becoming seriously ill. In the case of both, diarrhœa was well marked, and numerous cholera vibrios were present in the stools, though toxic symptoms were proportionately little pronounced. Metchnikoff also, by experiments on himself and others, obtained results which convinced him of the specific relation of the cholera vibrio to the disease. Lastly, we may mention the case of Dr. Örgel in Hamburg, who died from the disease in the course of experiments with the cholera and other vibrios. This took place at a time when there was no cholera in Germany. As the result also of observations on cholera epidemics and of cholera carriers, it may be concluded that only a certain proportion of people are extremely susceptible to cholera.

Toxins.—The general statement may be made that filtered cholera cultures as a rule have little toxic action—that is, comparatively little exotoxin is produced by the organism. It was, however, shown by R. Pfeiffer that the dead organisms were highly toxic, and that they produced, on injection into guinea-pigs, the same phenomena as living cultures, profound collapse with subnormal temperature being a prominent feature. Pfeiffer considered that the toxic substances are contained in the bodies of the organisms—that is, they are *endotoxins*—and that they are only set free by the disintegration of the latter. He showed also that when an animal is inoculated intraperitoneally with the cholera organism, and then some time later anti-cholera serum, which produces bacteriolysis, is injected, rapid collapse with a fatal result may ensue, apparently due to the liberation of the endotoxins. Dead cultures administered by the mouth produced no effect unless the intestinal epithelium was injured, in which case poisoning might result. Pfeiffer found that the toxic substances were to a great extent destroyed at 60° C., but even after heating at 100° C. a small proportion of toxin remained, which had the same action. Later, Macfadyen observed that the product obtained by grinding up the organisms frozen by means of liquid air, had a very high degree of toxicity

when injected intravenously. Like Pfeiffer, he found that the "endotoxin" was in great part destroyed at 60° C.

On the other hand, certain observers (Petri, Ransom, Klein, and others) have obtained toxic bodies from *filtered cultures*. Metchnikoff, E. Roux, and Taurelli-Salimbeni demonstrated the formation of such diffusible toxic bodies in fluid media. By means of cultures placed in collodion sacs in the peritoneum of animals, they found that the living organisms produce substances which diffuse through the wall of the sac and cause toxic symptoms. By greatly increasing the virulence of the organism, then growing it in broth and filtering the cultures on the third and fourth day, they obtained a fluid which was highly toxic to guinea-pigs (the fatal dose usually being 0·2 c.c. per 100 grams weight). The symptoms closely resembled those obtained by Pfeiffer. They found that the toxicity of the filtrate was not altered by boiling—apparently this toxic substance was different from Pfeiffer's endotoxin. Huntemüller obtained from various strains a rapidly acting extracellular toxin which was very labile and which he believed to be identical with the hæmolysin. He obtained an antitoxin to this toxin. The diversity in the results obtained by various workers seems only explicable on the assumption that different strains vary greatly as regards production of extracellular toxin. (See also El Tor vibrio, p. 573.)

Immunity.—As this subject is discussed in Chapter VI., only a few facts will be here stated, chiefly for the purpose of making clear what follows with regard to the means of distinguishing the cholera vibrio from other organisms. The guinea-pig or any other animal may be easily immunised against the cholera organism by repeated injections (conveniently made into the peritoneum) of non-fatal doses of dead vibrios; later, the living organisms may be used. In this way a high degree of immunity against the organism is developed; and further, the blood serum of an animal thus immunised (anti-cholera serum) has markedly protective power when injected, *e.g.* intraperitoneally, even in a small quantity, into a guinea-pig along with five or ten times the fatal dose of the living organism. Under these circumstances the vibrios undergo a granular transformation and, ultimately, solution; this phenomenon is generally known as Pfeiffer's reaction, and was applied by him to distinguish the cholera vibrio from organisms resembling it. The following are the details:

Pfeiffer's Reaction.—A loopful of an eighteen-hours' virulent agar culture of the organism to be tested is added to 1 c.c. of ordinary broth containing 0·001 c.c. of potent anti-cholera serum. The mixture is then injected into the peritoneal cavity of a young guinea-pig (about 200 grams in weight), and the peritoneal fluid of this animal (conveniently obtained by means of a 1 c.c. syringe) is examined microscopically after a few minutes and again after

twenty, forty, and sixty minutes. If the organisms injected are cholera vibrios, it will be found that they become motionless, swell up into globules, and ultimately break down and disappear—*positive result.* If they are found active and motile, then the possibility of their being true cholera organisms may be excluded—*negative result.* In the former case (positive result) there is, however, still the possibility that the organism has been destroyed by the normal peritoneal fluid. A control experiment should be made with 0·01 c.c. of normal serum in place of the anti-cholera serum. If no alteration of the organism occurs with its use, then the conclusion is that a true reaction has been given—the control animal will die from the infection in about twenty-four hours. Corresponding bacteriolytic effects may be obtained by *in vitro* methods, introduced since Pfeiffer's original method

The serum of an animal immunised by the above method has also marked agglutinating and other antibacterial properties (p. 232) against the cholera vibrio, and these properties closely correspond with Pfeiffer's reaction as regards specificity. For the serological identification of *V. choleræ* the agglutination reaction is now used as in the case of *B. typhosus*. It has been noted that along with *V. choleræ* there may occur in the stools of cholera cases vibrios which fail to agglutinate with an anti-cholera serum (Crendiropoulo, Greig). The relationship of these organisms to *V. choleræ* has not been finally settled, though some workers have claimed that the latter, under certain conditions, may undergo such alteration as to become inagglutinable by anti-cholera serum, *e.g.* in the bowel of convalescent patients and in water (Tomb and Maitra). Recent observations on antigenic variation offer some explanation of these findings (*vide infra*). It has been pointed out by Doorenbos that under the action of a bacteriophage, *V. choleræ* may become inagglutinable and acquire hæmolytic properties. It has also to be noted that strains when first isolated may be inagglutinable, but after repeated culture react to an anti-cholera serum.

An anti-cholera serum has little protective effect against the toxic action of the dead vibrios, and Pfeiffer maintained that little or no antitoxin to the endotoxin can be produced. On the other hand, Macfadyen, by injecting the endotoxin derived from the vibrios by grinding, obtained a serum which had antitoxic as well as agglutinating and bacteriolytic properties. Metchnikoff and others also obtained antitoxic sera for the extracellular toxins which they had prepared.

The *serum of cholera convalescents* has been found to possess protective and increased bactericidal action. These properties of the serum may be present eight or ten days after the attack of the disease, but are most marked four weeks after; they

then gradually diminish. Specific agglutinins appear in the serum of cholera patients, as in other diseases. They are most marked in convalescence, reaching the maximum in from two to three weeks from the onset of the disease, the serum then agglutinating in a dilution of 1 : 400 or even 1 : 1000 (Greig), Agglutinins are also often present in the blood of carriers. It should, however, be noted that normal serum may sometimes have an agglutinating effect on the cholera organism in dilutions up to 1 : 20.

For therapeutic purposes several *antisera* which are supposed to be antitoxic as well as antibacterial have been used. Reports regarding the effects of these sera are of conflicting character, but it cannot be said that they have a markedly beneficial action. The possession of marked antitoxic properties by these sera has not been established experimentally.

Cholera Bacteriophage.—A bacteriophage which is active towards the cholera vibrio has been obtained by various workers, and a method for its isolation from the stools of cholera cases was first described by d'Herelle. D'Herelle and Malone endeavoured to correlate the activity of this bacteriophage in the intestine of cases with recovery from the disease, and stated that in India outbreaks of cholera can be controlled by the addition of bacteriophage to water supplies. Bacteriophage preparations were also applied in the treatment of cases. D'Herelle and his co-workers claimed that if the bacteriophage is administered sufficiently early in the illness a considerable reduction in mortality results.

The study of the cholera bacteriophage and its practical application in prevention and treatment has been extended by Asheshov and by Morison. Asheshov pointed out the existence of different types of phage each producing different forms of "clearings" or "plaques" on cultures of the vibrio, and by repeated isolation from individual plaques succeeded in separating "pure-line" strains. Three types (A, B, and C) were first recognised; A acted only on the "smooth" form of the cholera vibrio, B on both "rough" and "smooth" and C almost exclusively on the "rough" variety, and these types were shown to be antigenically distinct. Further, vibrios which became resistant to one type were still sensitive to the remaining types. It was also noted that the lysis produced by a "pure-line" phage was invariably followed by secondary growth, whereas the several types when used in combination tended to prevent this. Thus the principle was adopted in using phage for the treatment of cholera to combine all available types acting on the vibrio and to incorporate in a preponderating proportion one which acted quickly, the others serving to prevent secondary growth. In order to determine the type of an unknown phage, growths resistant to each of the standard types, as well as one resistant to the phage under examination, are spread separately on culture medium; then there are superimposed on each "spreading," also separately, lysed filtrates containing the respective standard types and the phage in question. The results with the unknown culture and phage are compared with the standards. Morison has demonstrated nine different types of cholera phage

and has shown that by combining different types complete lysis of a cholera culture is produced without any subsequent secondary growth. He has applied practically such combined phages for the control of cholera in Assam, the material being distributed on a large scale and taken by mouth. Prophylactic and therapeutic effects have been claimed, as well as control of the spread of the disease.

Allied Vibrios.—*El Tor Vibrio.*—In 1905 Gotschlich obtained six different strains of a vibrio which conformed in cultural and serum reactions to *V. choleræ*, but differed from the classical cholera organism in having marked hæmolytic action, and also in producing a rapidly acting extracellular toxin. The organisms were obtained at El Tor from the intestines of pilgrims who had died with dysenteric symptoms, and there were no cases of cholera in the vicinity. There has been some difference of opinion as to whether these organisms are to be regarded as a distinct species or as variants of the cholera vibrio. The persons from whom they were isolated may possibly have been cholera carriers.

Paracholera Vibrios.—More recent observations have shown that there occur groups of cases with choleraic symptoms or merely diarrhœa, in which the vibrios present differ in certain respects from the cholera organism. Such cases have been studied by various workers (Castellani; Chalmers and Waterfield; Mackie and Storer), and the term paracholera has been applied. To speak generally, the symptoms are milder than those of true cholera, fatal results being comparatively rare, and the infection does not tend to spread as an epidemic. In addition to being found in cases of the disease, similar organisms have been obtained from the stools of contacts—that is, carriers occur. In those affected, the vibrios are often present in the stools in large numbers, and on isolation are found to have the morphological and cultural characters of the cholera organism; they are also virulent to the guinea-pig on intraperitoneal injection, and to the rabbit on intravenous injection, as in the case of the classical *V. choleræ*. They are, however, markedly hæmolytic, when tested both on blood agar plates and with suspensions of red corpuscles. Further, they differ serologically from the cholera organism even after repeated subculture—when tested in the form of suspensions of agar cultures they are not agglutinated at 37° C. by an anti-cholera serum, and they react negatively in Pfeiffer's reaction. Antisera for these organisms do not agglutinate the typical cholera vibrio. They also differ serologically amongst themselves, and several varieties may be distinguished in this way (Mackie).

Similar organisms have been isolated from water supplies (Greig). It seems likely that there exists a group of vibrios all resembling one another in general biological characters but serologically diverse and also varying in their parasitism and pathogenicity. *V. choleræ* is apparently the most highly parasitic type. Some of these vibrios may be purely saprophytic, occurring in water supplies in certain regions of the world or establishing themselves as commensals in the bowel. Others may occupy an intermediate position and possess potentially pathogenic properties, *e.g.* the paracholera vibrios.

In addition to the paracholera organisms described above, other vibrio types have also been isolated from choleraic cases, e.g. *V. metchnikovi, V. proteus,* phosphorescent vibrios, etc. No definite statement can be made as to the pathological relationships of these types in the human subject.

Recently Taylor, Pandit and Read have reported the results of an investigation in India of a large series of vibrio strains which did not agglutinate with anti-cholera serum. These were isolated from cases clinically resembling cholera, healthy persons and water. They formed a heterogeneous group both serologically and biochemically. Thus, thirty-one different serological types were recognised. Biochemically they were classified according to Heiberg's criteria (fermentation of mannose, saccharose and arabinose), along with the cholera-red and Voges-Proskauer reactions. Taylor and his co-workers had previously confirmed Heiberg's finding that true cholera vibrios ferment mannose and saccharose but not arabinose. While cholera-red-positive and -negative inagglutinable strains were generally positive and negative respectively in the Voges-Proskauer test, *V. choleræ* was found to be negative in the latter reaction. These workers have concluded that in India generally no vibrio of serological characters other than the classical is responsible for epidemics of cholera.

Antigenic structure of the cholera vibrio and allied organisms — As pointed out above, strains of the cholera vibrio isolated from the typical epidemic disease usually show a high degree of specificity in their agglutination reactions when tested by the ordinary methods, and in this way they have been clearly distinguished from " cholera-like " and paracholera vibrios (Greig, Mackie and others, *vide supra*). The El Tor vibrio has usually been found to behave like the true cholera vibrio in serological reactions though differing from it in being actively hæmolytic. The agglutination reactions of these organisms have generally been carried out with saline suspensions of agar cultures, incubation being at 37° C. for a short period. Douglas, using formolised suspensions and incubating at 50° to 55° C., elicited a group effect between *V. choleræ*

and a paracholera vibrio, and the different results obtained by the two methods were later emphasised by Mackie who showed that the essential serological distinction between *V. choleræ* and paracholera vibrios was more clearly demonstrated by the former technique. Recent work has shown that the cholera vibrio, like other flagellate bacteria, possesses antigenic constituents associated respectively with the flagellum and body of the organism (Balteanu and others). Gohar pointed out that while "cholera-like" vibrios may contain an antigenic component in common with *V. choleræ*, all strains of the latter organism possess the same flagellar (H) and somatic (O) antigens. Abdoosh found that some hæmolytic vibrios are agglutinated by an anti-cholera serum in virtue of their thermolabile (H) antigen, though differing from *V. choleræ* as regards their thermostable (O) component. These findings have shown that inter-relationships may exist between *V. choleræ* and certain other vibrios through common antigenic factors. This work has been extended by Gardner and Venkatraman, who suggest a classification of vibrios according to their H and O antigens. They recognise a "cholera group" of biochemically similar vibrios possessing a common H antigen and they divide this into subgroups each with a specific O constituent: I, comprising the classical *V. choleræ* and the El Tor vibrio; II, III, etc. which are mostly hæmolytic and include the various races of paracholera vibrios, "cholera-like" vibrios and also some strains classified as El Tor vibrios. This subdivision is substantially the same as that of earlier workers. The coagglutination or group reactions demonstrated by Douglas and by Mackie (*vide supra*) may now be interpreted as an expression of the H-antigenic relationship of these organisms, and apparently the original technique reveals the O agglutination effect and accentuates the serological distinction between *V. choleræ* and closely allied forms. The antigenic constitution of these organisms is, however, of considerable complexity and in addition to the specific O factor a non-specific component of the same type is also demonstrable. Further, White has observed that the transformation to the rough (R) form is associated with a loss of the specific O antigen, and the non-specific O component then becomes as it were unmasked. White has also described variants (designated ρ) which have lost their "rough" O antigen with the unmasking of another common constituent. The chemical nature of these components has been studied by Linton and his co-workers in India. They find that specificity depends on protein and carbohydrate substances and they have put forward a separate classification based on chemical constitution. The inter-relationship on the one hand and differences on the other among these organisms require further study. Variation also adds to the difficulties of classification; thus Linton and Mitra have stated that dissociation of a typical cholera strain may yield a derivative whose serological characters differ from the parent strain in virtue of changes in its chemical structure. So far the newer methods of antigenic analysis, while throwing light on some aspects of the serology of vibrios, have not added substantially to our original knowledge of their biological classification.

Anti-Cholera Vaccination.—Preventive inoculation against cholera first attracted attention largely as the result of Haffkine's

work in India. On the analogy of Pasteur's anti-anthrax inoculation, injections of attenuated organisms were administered first of all, and were followed by the injection of cultures of organisms of exalted virulence which had been passed repeatedly through the peritoneal cavity of guinea-pigs. Killed suspensions of the organism prepared from cultures on agar have more recently been extensively used as vaccines by Kolle and others. They are prepared according to the general technique described on p. 158. Adequate dosage of the vaccine is essential—two injections of 500 and 1000 million organisms at an interval of a week. The effect of the preventive inoculation of troops exposed to cholera infection in time of war, both in reducing very greatly the incidence and also in lessening the mortality of the disease, has been strikingly illustrated (Savas, Cantacuzène; Greenwood and Yule). The results of field tests of anticholera vaccination in India have been reported by Russell along with careful statistical analyses of records of cholera attacks and deaths among immunised persons and controls. Both oral and subcutaneous methods of administration were investigated, Besredka's cholera bilivaccine being used in the former case. Russell has concluded that a high degree of immunity is conferred by both methods but that the effect of subcutaneous vaccination is superior to that of the orally administered vaccine.

Methods of Diagnosis.—In the first place, the stools may be examined microscopically. Dried film preparations should be made and stained by any ordinary stain, though carbol-fuchsin diluted four times with water is specially to be recommended. Hanging-drop preparations should also be made, by which method the motility of the organism can be readily seen. By microscopic examination the presence of vibrios will be ascertained, and an idea as to their number obtained. In some cases the cholera vibrios are so numerous in the stools that a picture is presented which is obtained in no other condition, and a microscopic examination may be sufficient for practical purposes. According to Koch, a diagnosis was made in 50 per cent. of the cases during the Hamburg epidemic by microscopic examination alone. In the case of the first appearance of a cholera-like disease, however, all the other tests should be applied before a definite diagnosis of cholera is made.

If the organisms are very numerous, plates of Dieudonné's medium (p. 70) may be inoculated at once and a pure culture obtained from one of the colonies.

If the vibrios occur in comparatively small numbers, the best method is to inoculate peptone solution (1 per cent.) standardised to pH 8·0–9·0, and incubate for six to eight hours. At the end of that time the vibrios will be found on microscopic examination in large numbers in the surface pellicle, and thereafter plate cultures can readily be made on Dieudonné's medium. If the vibrios are very few in number, the peptone solution which has been inoculated

should be examined at intervals till vibrios are found microscopically. A second tube of peptone solution should then be inoculated and subcultures made later on Dieudonné plates. Though Dieudonné's medium in virtue of its alkalinity restrains the growth of most other intestinal bacteria and thus yields, as a rule, a practically pure growth of *V. choleræ*, in all cases the purity of the strain must be ensured by isolating from single colonies.

When a vibrio has been obtained in pure condition by these methods it should be tested, as regards agglutination, with a high titre anti-cholera serum. If it reacts positively it may be accepted for practical purposes as the cholera organism. At the same time the cultural characters and the hæmolytic and pathogenic properties may be tested. If it reacts negatively with anti-cholera serum it may be one of the para-cholera group, and similar tests should be made. At post-mortem examination of a suspected case portions of the unopened bowel some inches long at the middle of the ileum and just above the ileocæcal valve should be ligatured off and transmitted for bacteriological examination immersed, if necessary, in sterile saline.

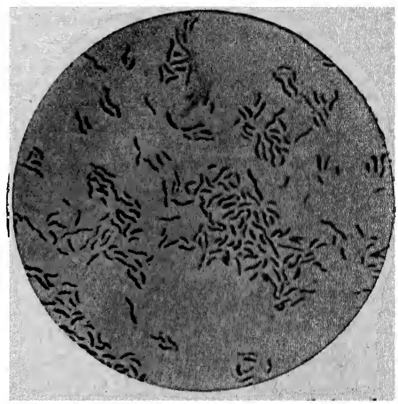


FIG. 121—Finkler and Prior's vibrio; from an agar culture of twenty-four hours' growth.
Stained with carbol-fuchsin. ×1000.

Other Vibrios.—The cholera vibrio belongs to a group of organisms which resemble it closely in microscopic and cultural characters. Some of these have been found to produce disease in animals, e.g. *V. metchnikovi* originally isolated from an epidemic disease of fowls; some again have been recovered from water or materials suspected of harbouring the cholera organism, *e.g.* Finkler and Prior's vibrio obtained from decomposing fæces from a case of *cholera nostras*; while others have been derived from various sources, *e.g.* Deneke's vibrio recovered from cheese. Before the importance of serological tests for the identification of *V. choleræ* was established, various cultural and biological characters were relied on for distinguishing them. Thus while Metchnikoff's vibrio closely resembles the cholera organism both in cultural appearances and in giving the cholera-red reaction, it can be readily distinguished from the latter by the effects of inoculation in animals, especially in pigeons and guinea-pigs. Subcutaneous inoculation of small quantities of pure culture in pigeons is followed by septicæmia, which produces a fatal result usually within twenty-four hours. Inoculation with the same quantity of the cholera vibrio produces practically no result; even with large quantities death is rarely produced. Metchnikoff's vibrio produces somewhat similar effects

in the guinea-pig to those in the pigeon, subcutaneous inoculation being followed by extensive hæmorrhagic œdema and a rapidly fatal septicæmia. Young fowls can be infected by feeding with virulent cultures. Organisms which are apparently the same as the *Vibrio metchnikovi* have been cultivated from water and from choleraic cases. Finkler and Prior's (Fig. 121) and Deneke's organisms liquefy gelatin rapidly and do not give the cholera-red reaction. Certain vibrios isolated from water are phosphorescent in cultures growing at 22° C. Phosphorescent vibrios have also been found in the stools of cholera-like cases. None of these organisms are agglutinated by an anti-cholera serum. Anaerobic vibrios have been isolated from the mouth and upper respiratory passages.

Vibrio fœtus.—This organism was described by Theobald Smith as the causal organism of a form of infectious abortion of cattle. It is somewhat variable in morphology, and may appear either as a typical vibrio about 2 μ in length or as a spirillum with 2 to 4 complete coils. It is motile and has a flagellum at one or both ends. The staining reaction is Gram-negative. In cultural characters it is micro-aerophilic, and when first isolated can only be cultivated with difficulty. The addition of a few drops of defibrinated horse blood to the condensation water of an agar slope permits growth in the fluid. After repeated subculture a delicate surface growth may be obtained. The organism does not exhibit any fermentative properties.

CHAPTER XVII

BACILLUS INFLUENZÆ AND ALLIED ORGANISMS, BACILLUS PERTUSSIS, BACTERIUM PNEUMOSINTES, AND STREPTOBACILLUS MONILIFORMIS

Bacillus Influenzæ

Authentic European records over nearly two centuries show that at varying intervals there have occurred severe and widespread epidemics of a condition which, in the description of its clinical features, conforms to the syndrome now designated influenza. Such epidemics have shown a tendency to pandemic spread, and this is an outstanding epidemiological feature of the disease. Since the period of bacteriological investigation two such pandemics have occurred (1889–92 and 1918–21) and their etiology has been extensively studied. The disease exhibits an exceptionally marked degree of infectiousness and assumes a high incidence in any population in which it occurs, spreading with great rapidity. The first accounts of the organism now known as the influenza bacillus were published simultaneously by Pfeiffer, Kitasato, and Canon, in January 1892. The two first-mentioned observers found it in the bronchial sputum, and obtained pure cultures, and Canon observed it in the blood in a few cases of the disease. This organism, up to the time of the recent pandemic of 1918, was generally accepted as the primary causal agent, although absolute proof was wanting. As a result of the 1918 pandemic, the etiology of the disease was extensively restudied, and opinions have varied as to whether this organism represents the primary agent or only an associated and secondary infection. In fact recent work indicates that the disease is primarily due to a filterable virus. This question will be discussed in Chapter XXV.

Bacillus Influenzæ (*Hæmophilus influenzæ*). — *Microscopical Characters.*—The influenza bacilli as seen in the sputum are very minute rods not usually exceeding 1·5 μ in length and 0·3 μ in thickness (Fig. 122). They are straight, with

rounded ends, and sometimes stain more deeply at the extremities. The bacilli occur singly, in pairs, or form clumps by their aggregation. They take up the basic aniline stains somewhat feebly, and are best stained by a weak solution (1 : 20–1 : 200) of carbol-fuchsin applied for five to ten minutes. They are Gram-negative, non-motile, and do not form spores. It must be recognised that the organism tends towards pleomorphism, and some strains show elongated thread-like forms which may even be the predominant morphological type. Certain strains may also exhibit spherical forms measuring 2 or 3 μ in diameter. Sometimes the filamentous forms show large globular or fusiform expansions. While strains recently isolated from influenza cases usually exhibit the short bacillary form, after artificial culture they tend to become atypical and to show morphological variation, which may be associated with changes in colony form (*vide infra*). In the early stages of the more acute cases with catarrhal complications of the respiratory system, influenza bacilli are frequently present in large numbers and may be easily found. On the other hand, it is often difficult or impossible to find them, even when the symptoms are severe.

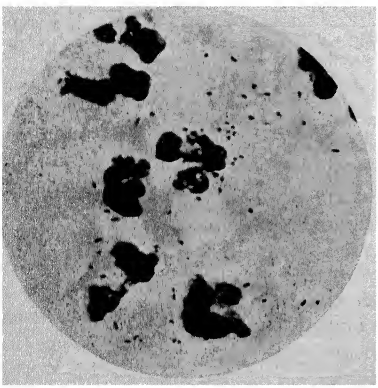


Fig. 122.—Film preparation of influenza sputum, showing influenza bacilli along with leucocytes. ×1000.

Cultivation.—One of the best media for the growth of the influenza bacillus is agar containing heated blood (see p. 69). Pfeiffer originally used blood-smeared agar for the cultivation of the organism. He had obtained growths of the bacilli on agar which had been smeared with influenza sputum, but subcultures on ordinary agar media or serum failed to grow. Considering the growth in the first cultures to be probably due to the presence of certain organic substances in the sputum, he tried, with success, the expedient of smearing the agar with drops of blood before making the inoculations. The blood of various animals is suitable, as well as human blood. Hence *B. influenzæ* and closely allied organisms have been designated

hæmophilic. The growth of the influenza bacilli on blood agar, incubated at 37° C., appears within twenty-four hours, in the form of minute circular colonies, almost transparent like droplets of dew (Fig. 123). When numerous, the colonies are scarcely visible to the naked eye, but when sparsely arranged they may reach the size of a pin's head. This size is generally reached on the second day. The colonies do not show any special features; normally, they are of the "smooth" type, but variation to the "rough" form may occur. In cultures the bacilli may show considerable variations in size (Fig. 124) and in shape, and after a time involution forms (Fig. 125) may be present; they die out somewhat quickly, and in order to keep them alive subcultures should be made every four or five days

FIG. 123—Colonies of influenza bacilli on blood-agar plate. (There are a few larger colonies of other organisms.) ×5.

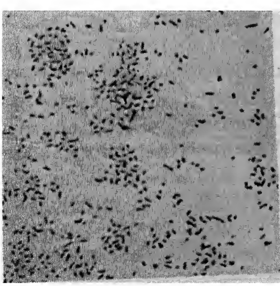


FIG. 124.—Film preparation from young culture of influenza bacillus. Stained with weak carbol-fuchsin. ×1000.

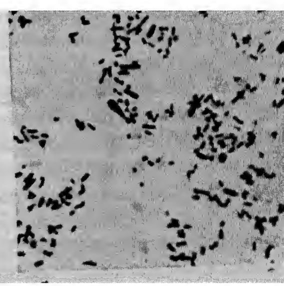


FIG. 125—Film preparation from older culture of influenza bacillus, showing involution forms. ×1000.

until the organism is accustomed to culture medium. Even on subculture, growth on the ordinary agar media is practically absent. Growth is favoured by symbiosis: thus, in a mixed plate culture it may be noted that the growth of *B. influenzæ*

is more abundant in the neighbourhood of colonies of other organisms, *e.g.* staphylococcus; this has been designated "satellitism." In blood-broth cultures a thin whitish deposit forms at the bottom of the container. The limits of growth are from 25° to 42° C., the optimum temperature being that of the body. The influenza bacillus flourishes much better under aerobic than anaerobic conditions.

Owing to its peculiar hæmophilic character *B. influenzæ* has been carefully studied in recent years as regards its requirements for growth in artificial culture, and interesting data have been obtained. Apparently this organism will only flourish in the presence of two growth-promoting principles. One of these, designated the X factor (Avery), is a highly thermostable substance which remains unaltered even after autoclaving (*e.g.* at 120° C.). This principle is associated with hæmoglobin, and some workers regard it as hæmatin. It has been supposed that hæmatin acts as a peroxidase in catalysing oxidation by peroxides and so promotes the respiratory functions of the organism. Certain iron compounds, however, have been found to serve as an X factor without exerting peroxidase properties (Baudisch). On the other hand, these may possess catalase functions. It has also been claimed that under anaerobic conditions the organism can dispense with the X factor (Anderson) and the question therefore arises whether the growth-promoting effect of this factor may be due to catalase action protecting the organism from peroxide (*vide* p. 20). The other factor (V) is less thermostable, being destroyed at temperatures above 100° C., and it has been suggested that it may be of the nature of a vitamin. Blood agar contains both these growth-stimulating substances. Some vegetable tissues, *e.g.* potato, also supply them. Thjøtta has shown that the V factor is contained in red cells, bacteria, yeasts, and vegetables. It is synthetised by certain bacteria, *e.g.* staphylococci, and this explains the symbiotic relationship of *B. influenzæ* to other organisms (*vide supra*).

Certain artificial media have proved specially valuable for the isolation and growth of the bacillus. The oleate-blood-agar of Avery [1] favours the growth of *B. influenzæ* and inhibits that of certain other organisms frequently present in sputum,

[1] 5 c.c. of a sterilised 2 per cent. solution in water of sodium oleate and 1 c.c. of a sterile suspension of rabbit's blood cells (prepared by centrifuging defibrinated blood, removing the serum, and making up to original volume with sterile broth) are added to 94 c.c. of melted nutrient agar at 90° C. The agar should have a pH of 7 to 7·4.

e.g. streptococci and pneumococcus. The so-called "chocolate" agar, prepared by adding blood to nutrient agar at a temperature of 90° C., yields an abundant growth of *B. influenzæ*, and cultures are somewhat more luxuriant on this medium than on agar containing unaltered blood. It has been supposed that the difference between the altered and unaltered blood depends on the fact that the fresh blood deviates oxygen from the bacillus in virtue of its greater oxygen affinity (Fildes). Another medium particularly valuable for isolating the organism is agar with a peptic digest of blood added [1]; it has the advantage of being transparent as compared with "chocolate" agar.

Some strains produce hæmolysis when growing on blood agar, and it has been noted that these show the atypical thread-like forms referred to above; the colonies are also more opaque than the common type, and in fluid medium a flocculent growth results. Most of the hæmolytic strains differ from the typical form of *B. influenzæ* in being independent of the X factor for their growth and requiring only the V factor. Strains with similar growth requirements, but lacking hæmolytic power, have been isolated from ulcerative endocarditis. Fermentation reactions are somewhat irregular: glucose is generally fermented with acid production; lactose and mannitol are not acted on; galactose and lævulose are generally fermented by the non-hæmolytic strains, though less frequently by the hæmolytic organisms. Saccharose fermentation is a more frequent property of the hæmolytic type than the non-hæmolytic strains. Fermentation reactions may, however, vary on continued cultivation. Cultures of the influenza bacillus exhibit reducing properties and nitrite is formed from nitrate. A considerable proportion of strains of *B. influenzæ* have been found to produce indole in culture; others, including generally the hæmolytic variety, lack this property.

It has been generally agreed that strains of *B. influenzæ*

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[1] Peptic blood digest is prepared by keeping a mixture of 150 c.c. saline, 6 c.c. pure HCl, 50 c.c. defibrinated sheep's blood, and 1 gram pepsin, B.P. granulated, in a well-stoppered bottle at 55° C. in the water bath for two to twenty-four hours. Then add 20 per cent. NaOH solution (about 12 c.c. are required) till a sample of the mixture diluted with water gives a permanganate-red colour with cresol-red indicator. Now add pure HCl drop by drop till a sample gives almost no change of colour with cresol-red, but gives a red tint with phenol-red (avoid excess of acid). 0·25 per cent. of chloroform is added to the mixture and dissolved by shaking. This digest keeps for many months; before use the mixture should be shaken and 2 to 5 per cent. added with a sterile pipette to broth or agar (Fildes, *Brit. Journ. Exper. Path.*, 1925, vi. 62).

constitute a serologically heterogeneous group and represent a multiplicity of races. According to Jordan and Sharp, and others, strains may even exhibit serological individuality. This is analogous to what has been observed in the *B. coli* group (*vide* p. 512). Recently, however, it has been pointed out by Pittman in a study of certain "smooth" strains of *B. influenzæ* that these are capsulated and can be classified into two serological types, their specificity depending apparently on a capsular carbohydrate substance. Such strains on transformation to the "rough" form lose their original serological characters.

The powers of resistance of this organism are of a low order. Pfeiffer found that dried cultures kept at the ordinary temperature were usually dead in twenty hours, and that if sputum were kept in a dry condition for two days cultures could be no longer obtained. From these experiments it follows that outside the body in ordinary conditions they can remain alive only for a short time.

Distribution in the Body.—The bacilli are found chiefly in the respiratory passages in influenza. They may be present in large numbers in the nasal secretion, generally mixed with a considerable number of other organisms, but it is in the small masses of greenish-yellow sputum from the bronchi that they are present in largest numbers, in many cases almost in a state of purity. They occur in large clumps and in the early stages of the disease are chiefly lying free. As the disease advances, they may be found in considerable numbers within the leucocytes, and towards the end of the disease a large proportion have this position. They may persist for weeks after symptoms of the disease have disappeared, and may still be detected in the sputum. Especially is this the case when there is any chronic pulmonary disease. They occur also in large numbers in the capillary bronchitis and catarrhal pneumonia of influenza, as Pfeiffer showed by means of sections of the affected parts. In these sections he found the bacilli lying amongst the leucocytes which filled the minute bronchi, and also penetrating between the epithelial cells and into the superficial parts of the mucous membrane. McIntosh has shown that at a very early stage the bacilli are present in the upper respiratory tract but absent from the lung. Other organisms also, *e.g.* streptococci, pneumococci, may be concerned in the pneumonic conditions following influenza. Occasionally in the foci of suppurative softening in the lung the influenza bacilli have been found in a practically pure condition. In cases of empyema the organisms present

would appear to be chiefly streptococci and pneumococci, but influenza bacilli may also be present; whilst in the gangrenous conditions, which sometimes occur, a great variety of organisms has been found.

Pfeiffer's observations on a large series of cases convinced him that the organism was very rarely present in the blood—that in fact its occurrence there must be looked upon as exceptional. The conclusions of other observers have, on the whole, confirmed this statement. The bacillus may be present in other lesions complicating influenza. Pfeiffer found it in inflammation of the middle ear, and it has been observed in meningitis following influenza. Pfuhl considered that in these cases the path of infection is usually a direct one through the roof of the nasal cavity. He also found *post mortem*, in a rapidly fatal case with profound general symptoms, influenza bacilli in various organs, both within and outside of the vessels. In a few cases also the bacilli have been found in the brain and its membranes, with little tissue change in the parts around.

Extensive observations on the bacteriology of the respiratory system show that bacilli like *B. influenzæ* may be present in a great variety of conditions; and though these may represent a biological group possessing somewhat different degrees of pathogenicity, it is impossible to differentiate them from strains isolated from influenza. These bacilli have been obtained from the fauces, bronchi, and lungs in various inflammatory conditions, and also in specific fevers. They are not infrequent in phthisis, in bronchiectatic cavities, and in chronic inflammation of the nasal sinuses. They may be present in pure culture in suppuration in a joint, especially in children. They may also be recovered frequently from the nose and throat in quite healthy persons.

A hæmophilic organism has been not infrequently found in cases of acute primary meningitis occurring in young subjects, *e.g.* from two to three years of age (p. 361). In the cerebro-spinal fluid this organism may appear as somewhat elongated filaments, and it was originally thought to be of the nature of a leptothrix. It is, however, indistinguishable from the influenza bacillus, and the filamentous form is not a constant character. This organism differs from typical strains of *B. influenzæ* in its high virulence for rabbits (Rivers).

B. hæmoglobinophilus canis isolated from the preputial sac of dogs is of interest in its relationship to *B. influenzæ*, which it resembles in general characters. For its growth, however, this organism requires only the X factor, being independent of the V substance.

B. influenzæ suis is the designation given to strains isolated from swine influenza in which they have been found associated with a filterable virus (*vide*, p. 771). While resembling *B. influenzæ* they are stated to lack fermentative properties.

It should be noted here that the *Koch-Weeks bacillus* (*vide* p. 314) is practically identical in biological characters with *B. influenzæ*.

As regards their requirement of the X and V factors, strains of *B. influenzæ* and allied organisms may be classified as follows :

	X	V factors.
Typical (Koch-Weeks bacillus included in this group)	+	+
Hæmolytic strains (also certain non-hæmolytic strains, *e.g.* from ulcerative endocarditis) .	−	+
B. hæmoglobinophilus canis	+	−

Experimental Inoculation.—Pfeiffer, by injecting living cultures of the organism into the lungs of monkeys, in three cases produced a condition of fever of a remittent type. There was, however, little evidence that the bacilli had undergone multiplication, the symptoms being apparently produced by their toxins. He accordingly came to the conclusion that the influenza bacilli contain toxic substances which can produce in animals some of the symptoms of the disease, but that animals are not liable to infection, the bacilli not having the power of multiplying to any extent in their tissues. Wollstein distinguished virulent and avirulent types according to the result on intravenous injection in the rabbit ; the virulent types caused death in about twenty-four hours, the bacilli being numerous in the blood. The dose used, however, was comparatively large, namely, a blood-agar slope culture for a rabbit of 1000 grams. Strains from the respiratory tract were non-virulent by this test ; those from the blood and meninges, and rarely from pneumonic lung, were virulent. Wollstein found that a fatal cerebro-spinal meningitis could be produced in monkeys by the subdural injection of virulent cultures. McIntosh has claimed that *B. influenzæ* produces a diffusible toxin, and that inoculation of animals, *e.g.* rabbits and guinea-pigs, with filtered cultures leads to pathological changes in the lung which show a close resemblance to those observed in man. This toxic substance is not to be regarded as a true exo-toxin. Blake and Cecil produced an influenza-like illness in monkeys by inoculation with *B. influenzæ* cultures. Cecil and Steffen, by introducing virulent influenza bacilli into the throat and nose in the human subject, produced an acute catarrhal infection of the upper respiratory passages, which, however, fell short of typical influenza.

Reviewing the extensive experimental work that has now been carried out with *B. influenzæ*, we may say that conclusive proof of its primary etiological relationship to the disease has

not been obtained. The frequent association of the organism with the epidemic disease has been well established, but it is not invariably present. Its occurrence is, moreover, specially related to the catarrhal complications. During the 1918 pandemic, cases were met with in which the fever and general manifestations were exceedingly marked but unaccompanied by obvious inflammatory involvement of the respiratory tract, and *B. influenzæ* was not detectable in the nose and throat secretions. Even in cases with pulmonary complications, the organism could not be found constantly in the sputum when examined by the best methods. Such observations led to the view that, contrary to previous belief, this organism is not the primary agent in epidemic influenza, though it was admitted that it represents a frequent concomitant infection and may be responsible, like streptococci and the pneumococcus, for pulmonary complications.

In the last pandemic, preventive inoculation with vaccines of *B. influenzæ* along with other associated organisms, *e.g.* pneumococci, streptococci, was extensively applied. There has been no undoubted statistical proof of the efficacy of the procedure, and evidence from this source as to the etiological relationship of *B. influenzæ* to the epidemic disease is awanting. On the other hand, the observed relationships of the organism to lesions in the lungs and elsewhere leave no room for doubt that it is possessed of pathogenic properties.

The question of the virus etiology of influenza and the pathogenic association of a filterable virus and *B. influenzæ* is discussed in Chapter XXV.

Methods of Examination.—(*a*) *Microscopic.*—A portion of the greenish-yellow purulent material which often occurs in little round masses in the sputum should be selected, and film preparations should be made in the usual way. Films are best stained by dilute carbol-fuchsin (1 : 20—1 . 200), staining being prolonged for five to ten minutes. In sections of the tissues, such as the lungs, the bacilli are best brought out, as shown by Pfeiffer, by staining with the same solution as above for half an hour. The sections are then placed in alcohol containing a few drops of acetic acid, in which they are dehydrated and slightly decolorised at the same time. They should be allowed to remain till they have a moderately light colour, the time varying according to their appearance. They are then washed in pure alcohol, cleared in xylol, and afterwards mounted in balsam.

(*b*) *Cultures.*—A suitable portion of the greenish-yellow material having been selected from the sputum, it should be washed well in several changes of sterilised water. A loopful should then be used to make successive strokes on the surface of blood-agar plates. Heated-blood ("chocolate") agar or Fildes' peptic blood digest agar

are specially useful for isolating the organism. The plates should be incubated at 37° C., when the transparent colonies of the influenza bacillus will appear, usually within twenty-four hours. These should fail to grow in subculture on ordinary agar media. It has been found that a broth culture filtrate of a certain type of *Penicillium* can be used as a selective bacteriostatic agent in isolating *B. influenzæ*. It inhibits the growth of the Gram-positive cocci and, with some exceptions, also Gram-negative cocci. The filtrate ("penicillin") is incorporated in the medium or spread on the surface, *e.g.* two to six drops according to the potency of the filtrate (Fleming).

The requirement of the X and V factors can be determined by serial cultural tests in peptone water (1) *plus* hæmatin, (2) *plus* yeast extract and (3) *plus* both hæmatin and yeast extract.

Bacterium pneumosintes (Dialister pneumosintes)

In 1921 Olitsky and Gates described a supposed filter-passing virus in the naso-pharynx of cases of influenza. They stated that when inoculated intratracheally in rabbits it produced fever, leucopenia, hæmorrhages in the lung with œdema and emphysema. This agent could only be demonstrated in the earliest stage of the illness and was absent after thirty-six hours from the onset. It could not be isolated from other conditions or from healthy persons. When inoculated into rabbits, it apparently underwent increase in the lungs and could be passed through animals in series. This agent, derived from the naso-pharyngeal secretions and the lungs of experimental rabbits, could be passed through Berkefeld V and N filters, and withstood 50 per cent. glycerol for long periods. In subsequent studies of the virus, Olitsky and Gates identified it as an exceedingly minute bacilloid structure measuring 0·15 to 0·3 μ in length, which they designated *Bacterium pneumosintes*. Cultures from the filtrates were obtained in Smith-Noguchi medium (*vide* p. 672), and, when inoculated intratracheally in rabbits, reproduced the effects already described as following the inoculation of filtrates of the naso-pharyngeal secretion. Olitsky and Gates regarded the condition produced in animals by their filterable organism as analogous to the primary condition in epidemic influenza, and supposed that this was the causal organism which predisposes the lung tissue to secondary microbic invasion, with all the characteristic complications of the disease. The occurrence of a similar organism in influenza has been noted by others in different parts of the world (Gordon, Lister, and others). In more recent studies, Olitsky and Gates have shown that similar anaerobic filter-passing Gram-negative organisms may occur normally in the throat secretions. These have also been observed by Garrod and others, and it is now accepted that *Bacterium pneumosintes* has no definite relationship to epidemic influenza. It is doubtful if these organisms have any pathogenic role.

While the characteristic morphological appearances are those of a very small coccal or bacillary organism of the dimensions given above, it has been found that after continuous artificial culture the bacillary form becomes more marked, the length of the organism increasing to 0·5 or 1 μ. It may occur singly, in pairs, or even in short chains. It is best demonstrated in culture by staining with

polychrome methylene-blue. It can also be stained, however, with simple stains, and is Gram-negative. It is a strict anaerobe, and when first grown in Smith-Noguchi medium a cloudiness forms in the medium, starting after three to four days at 37° C., at the foot of the tube round the tissue fragment. Colony growths can be obtained under anaerobic conditions on blood agar. The colonies are very small and transparent and take several days to appear.

Streptobacillus moniliformis (Haverhillia multiformis)

This organism was isolated by Levaditi and his co-workers from the blood in cases of erythema multiforme and was described in America by Parker and Hudson as the causal agent of a condition

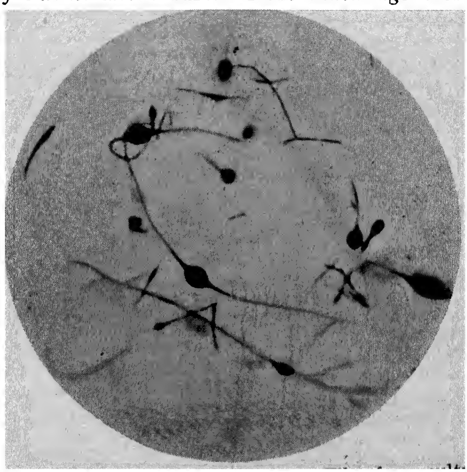


Fig. 126.—*Streptobacillus moniliformis.* Film from culture showing filamentous forms with fusiform expansions. Stained with dilute carbol-fuchsin. ×1000.

designated by them "Haverhill fever—*Erythema arthriticum epidemicum*" characterised by multiple arthritis and erythema. It has also been reported in epizootics in mice (Levaditi *et al*; Mackie *et al*; and Strangeways) and has been found in the nasopharynx of rats (Strangeways). It seems likely that this organism has been responsible for some cases of "rat-bite fever" (*vide infra*). In mice the organism produces a general infection and multiple arthritis. The joints of the feet and vertebræ are frequently affected and swellings of the feet, legs, and tail are noticeable. Paralysis of the hind legs may occur, apparently as a result of vertebral lesions. *Post mortem* there may be enlargement of the spleen, which shows areas of necrosis. The liver is sometimes similarly affected. Conjunctivitis and enlargement of lymph nodes are also common features.

Morphology.—The organism is exceedingly pleomorphic, occurring as short Gram-negative bacilli ($1–3\ \mu \times 0{\cdot}3–0{\cdot}4\ \mu$) or as elongated filaments which may show fusiform or globular expansions (Fig. 126); these are most frequently seen in recently isolated cultures. The morphological appearances are very similar to those of the influenza bacillus (*q.v.*). Some observers have described branching of the filaments and it is possible that streptothrix-like forms reported by Schottmuller and others in cases of rat-bite fever have been strains of this organism. While in culture the filamentous forms may suggest mycelium formation there is no true branching (van Rooyen).

Cultivation.—A high concentration of blood or serum is necessary for maximum growth on culture medium. Löffler's serum is specially suitable. Growth occurs aerobically and best at incubator temperature. The colonies are at first pin-point in size and do not as a rule exceed 1 mm. in diameter. Cultures die within two or three days and even after continued cultivation viability is feeble. Neither the X nor V factors of blood are required for growth, and in this respect the organism differs entirely from *B. influenzæ*.

Klieneberger has described in cultures of *Streptobacillus moniliformis* a symbiont which resembles the organisms of pleuro-pneumonia and agalactia. The symbiont observed by means of impression preparations of colonies, shows no bacillary structures but occurs in the form of filterable granules, filaments, and other structural elements resembling those of the pleuropneumonia organism (*vide* p. 484). Klieneberger has also reported the separation of the symbiont from cultures of *Streptobacillus moniliformis*. Van Rooyen who has studied the morphology of this organism in impression preparations has been unable to confirm the existence of an independent colonial system as described by Klieneberger, and has drawn attention to the occurrence in the organism of characteristic refractile granules which may also occur extracellularly. He states that they show a close similarity to the granular phase of the symbiont described by Klieneberger. It is difficult at present to interpret these findings. The frequent occurrence of the "pleuropneumonia-like" organism in cultures of *Streptobacillus moniliformis* might suggest that it is a derivative or phase of the latter. On the other hand, its isolation in independent culture points to its being a separate entity.

Experimental Inoculation.—Mice can be infected experimentally by inoculation with cultures, but different strains of these animals vary considerably in their susceptibility both to the spontaneous and the induced infection. The Simpson-March albino strain is specially susceptible. The ordinary hybrid black-coated stock mice are highly resistant. Successful inoculation leads to a rapidly lethal general infection or a subacute disease with localised lesions in bones and joints as in the spontaneous infection (*vide supra*).

Bacillus Pertussis (of Whooping-Cough)

Whooping-cough is now in many countries one of the most important epidemic diseases of childhood; in addition to

causing a considerable death-rate, it also leads to serious impairment of resistance to other infections.

Up to the year 1906, the chief result of bacteriological observations, of which those of Spengler, Krause and Jochmann, and Davis may be mentioned, had been to demonstrate the very frequent presence of minute hæmophilic bacilli resembling *B. influenzæ* in the sputum and also in the lesions in this disease. In that year, however, Bordet and Gengou published an account of another minute organism, designated *B. pertussis*, and brought forward certain facts which gave strong support to its etiological relationship.

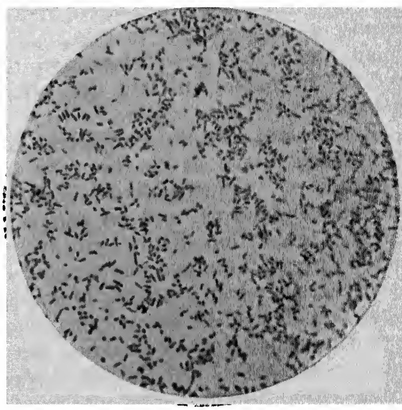


FIG. 127—Film preparation from a twenty-four hours' culture of the bacillus of whooping-cough (Bordet-Gengou). Stained with dilute carbol-fuchsin. ×1000.

Characters of the Bacillus Pertussis. (*Hæmophilus pertussis*).—The organism, as seen in the sputum, occurs in the form of minute oval rods scarcely larger than the influenza bacillus. They stain rather faintly with ordinary stains, and their margin and extremities are often more deeply coloured than the centre, which may appear as an uncoloured spot; they are Gram-negative and do not form spores. In cultures they present the same characters, and are distinctly less pleomorphous than the influenza bacillus (Fig. 127). They are specially numerous at the beginning of the disease, and they may be found in large numbers in almost pure culture in the opaque whitish sputum expectorated from the bronchi; as the disease advances they become scanty, and may disappear when the symptoms of the disease are still prominent. *B. pertussis*, like the influenza bacillus, has a tendency to localise in the ciliated epithelial lining of the bronchioles. The bacillus has not been found in the blood, unless as an agonal phenomenon (Klimenko).

Bordet and Gengou succeeded in obtaining pure cultures on the glycerol-potato-blood-agar medium described on p. 69, and this was found to be the most suitable of all the media tried. In

the first cultures, growth consists of characteristic minute, raised, "pearly" colonies; later it becomes much more abundant, and subcultures may also be readily made on ordinary blood- or serum-agar media. As compared with that of the influenza bacillus, growth after several generations is thicker and less transparent and the margins are more sharply marked off; it also presents a tenacious character. The presence of blood, though favouring the growth, is not so essential as in the case of the *B. influenzæ*, and *B. pertussis* can grow independently of the X and V factors (p. 582). The organism is a strict aerobe, and in the case of cultures in fluid media, *e.g.* serum broth, the tubes ought to be placed in a sloped position in order to expose a large surface to the air. *B. pertussis* does not ferment glucose or other carbohydrates and does not produce indole or reduce nitrate (cf. *B. influenzæ*). It is non-hæmolytic.

Bordet and Gengou completely confirmed the observations mentioned above as to the very frequent, almost constant, presence of "influenza-like" bacilli. They obtained growths of these organisms, and on comparing them with their own bacillus found that distinct cultural differences could be made out. The most marked distinctions were, however, obtained on studying the serum reactions of convalescents from the disease. They found that in many cases, though not invariably, such sera agglutinated their bacillus, but none of the "influenza-like" organisms. But the most important result was that in every case examined the serum of convalescents gave the complement fixation reaction very markedly with the whooping-cough bacillus, but with none of the others. This means, of course, that a specific antibody for the bacillus is present in the serum, and points to a true infection with the organism (p. 151). The results of the application of the test to adults suffering from bronchial irritation have been to show that they more frequently suffer from the infection than was formerly supposed, the paroxysmal stage being often absent.

It is specially noteworthy that this organism constitutes a homogeneous species from the serological point of view and contrasts in this respect with *B. influenzæ*. Thus most observers have found on testing the agglutination reactions of freshly isolated strains with antisera that they all react similarly. On the other hand, after cultivation, strains readily undergo antigenic variation. This may be analogous to the S→R transformation, though there is no obvious change in colony structure. Certain serological observations suggest that

the change in antigenic characters develops in successive phases (Leslie and Gardner; Toomey *et al*).

Pathogenic Effects.—The general results obtained by Bordet and Gengou showed that the ordinarily used animals were not susceptible to true infection with the bacillus, but that it contained a powerfully acting endotoxin, which produced both local and general effects. The injection of a small quantity of the bacillus into the eye of a rabbit produced a local necrosis, with little inflammatory change, and the introduction of dead, as well as living, cultures into guinea-pigs caused death from toxic action, there being hæmorrhagic œdema locally, and hæmorrhages and necrotic foci in organs. They advanced the view that the bacillus is present in large numbers at the beginning of the disease, and inflicts some local damage on the bronchial tubes which may persist after the disappearance of the bacillus, and keep up the irritation. Klimenko succeeded in infecting monkeys and young dogs by intratracheal injection of pure cultures of the bacillus. After a period of incubation, there occurred an illness in which symptoms of pulmonary irritation and irregular pyrexia were outstanding features. Usually, in the case of the dogs, a fatal result followed after two or three weeks, and *post mortem* there were found catarrhal changes in the respiratory tract and sometimes patches of broncho-pneumonia, from which the bacillus could be recovered in pure culture. The serum of the infected animals gave the complement fixation reaction. A specially interesting finding was that a number of healthy young dogs contracted the disease by contact with the inoculated. Fraenkel also obtained results closely similar to those of Klimenko. In recent years further attempts have been made to establish the etiological rôle of *B. pertussis* by experimental inoculation. Sauer and Hambrecht introduced cultures into the nares and larynx of young monkeys (e.g. *Macacus rhesus*) and in certain cases after an incubation period of one to three weeks paroxysmal coughing resulted. Examination of the blood revealed the transitory lymphocytosis which is characteristic of the human disease. *B. pertussis* was recovered by throat culture during life and from the lungs *post mortem*. Recovered animals were immune to subsequent inoculations. Shibley and Hoelscher claim to have reproduced the disease in chimpanzees. The production of whooping-cough in children by means of cultures of *B. pertussis* has also been recorded (Macdonald and Macdonald).

More recently, experiments have been carried out in chim-

panzees by Rich and his co-workers, which demonstrate the infectivity of cultures of *B. pertussis* and of unfiltered material from the respiratory passages of cases of whooping-cough. In these animals conditions closely resembling whooping-cough were produced. No evidence was obtained that any filterable infective agent is responsible for the complete syndrome of effects which is typical of whooping-cough, though there were some indications of the presence in experimentally inoculated animals of a transmissible filterable catarrh-producing agent.

The question has been considered whether a virus may be associated with *B. pertussis* in whooping-cough, and in this connection it is of interest that intranuclear inclusion bodies have been observed in the alveolar epithelium in the disease. The interstitial infiltration of the bronchial wall with mononuclear cells is a feature which provides an analogy with virus infections of the respiratory tract such as measles and influenza. The extremely infectious nature of the disease and the lasting immunity following recovery have also suggested the possibility of whooping-cough being of virus origin. So far, however, attempts to demonstrate a specific virus have been negative or inconclusive.

The original findings of Bordet and Gengou have received general confirmation. Sugare and McLeod, who have isolated *B. pertussis* from a series of cases of whooping-cough, obtained successful results in every instance on examination in the first week of the disease. They failed to find the organism in secretions from the larynx, trachea, bronchi, and lungs of fifty children under ten years of age who had died from diseases other than whooping-cough. It should be noted that while the organism may be detected in most cases during the early catarrhal stage, it is less regularly found in the expectoration during the paroxysmal stage and may be absent at a later period. Thus, as a rule, cases are non-infective after four weeks from the onset of the typical paroxysmal coughing (Kristensen). Though it is impossible to make a definite pronouncement on the subject, it may be said that there is strong evidence for the etiological relationship of Bordet and Gengou's bacillus.

Artificial immunisation with a *B. pertussis* vaccine has been advocated as a prophylactic measure. Several extensive investigations of its efficacy have now been carried out by Danish and American workers. The results point to its prophylactic value. In this connection the antigenic variation of laboratory cultures associated with loss of virulence must be

considered (*vide supra*). It is therefore essential that vaccines should be prepared from recently isolated virulent strains and not from stock cultures. 7–8 c.c. of a vaccine containing 10,000 million organisms per c.c. may be given in divided weekly doses (Sauer).

Methods of Examination.—A portion of tenacious sputum expectorated at the end of a paroxysm of coughing into a sterile Petri plate should be obtained, if possible at an early stage of the disease. Some of the thickest portion of the specimen is emulsified in sterile saline, and a loopful is spread over a plate of freshly prepared Bordet-Gengou medium. A second plate is inoculated with the undiluted sputum at the same time The plates are then incubated at 37° C. and examined after twenty-four, forty-eight, and seventy-two hours. Colonies of *B. pertussis* appear after seventy-two hours as small raised, pearl-like colonies which can be recognised by their characteristic features.

A useful procedure for obtaining direct cultures of the organism is to hold an open plate of the special medium about four inches in front of the mouth of the patient during the paroxysm of coughing. Fifteen seconds exposure is sufficient. The plate is then closed and incubated.

B. influenzæ and organisms of this type which may occur in sputum require careful differentiation from *B. pertussis* The strictly hæmophilic character of *B. influenzæ* and the features of the growth of *B. pertussis* after several subcultures, as compared with that of the former (*vide supra*), serve to distinguish the two organisms While *B. pertussis* may be grown after subcultivation on a serum medium, *B. influenzæ* completely fails to develop on such media. According to Sugare and McLeod, the chief features which distinguish the *B pertussis* from *B. influenzæ* are, in the case of the former, the slowness of growth ; the appearance of the colonies on Bordet-Gengou medium, which are raised and pearly ; the persistence of viability in cultures after keeping four to five weeks at 10°–0° C. ; and the absence of growth of recently isolated strains on heated blood agar ; also the very active catalase effect of cultures, whereas *B. influenzæ* gives this only slightly.

CHAPTER XVIII

BACILLUS PESTIS (OF ORIENTAL PLAGUE) AND ALLIED ORGANISMS (PASTEURELLA GROUP)

THE bacillus of Oriental plague or bubonic pest was discovered independently by Kitasato and by Yersin during the epidemic

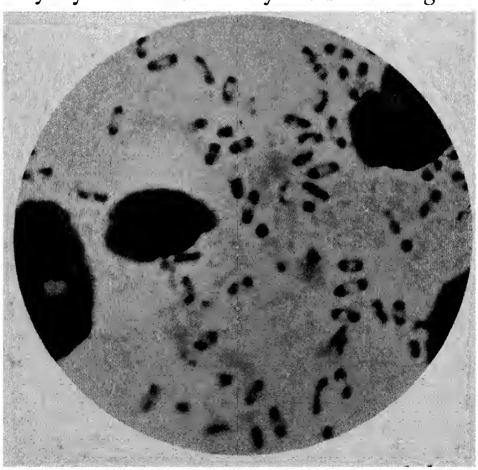


FIG. 128.—Film preparation from a plague bubo, containing enormous numbers of bacilli, most of which show well-marked bipolar staining.

Leishman's stain. ×2000.

at Hong-Kong in 1894. They cultivated the organism from a large number of cases of plague, and reproduced the disease in susceptible animals by inoculation of pure cultures. It is to be noted that during an epidemic of plague, sometimes even preceding it, a high mortality has been observed amongst certain wild rodents, especially rats and mice, and that from the bodies of these animals found dead in the plague-stricken

district, the same bacillus was obtained by Kitasato and also by Yersin. Thus it is now well established that plague is an epizootic disease of rats and various other rodents in certain parts of the world, and that the human disease originates from an animal source.

It should also be noted that *B. pestis* is one member of a fairly well defined group of bacteria (*Pasteurella* group) which includes the organisms of hæmorrhagic septicæmia of animals (e.g. *B. suisepticus* of swine plague) and *B. pseudotuberculosis rodentium* responsible for a tuberculosis-like disease of various rodents.

Bacillus Pestis (*Pasteurella pestis*).—*Microscopical Char-*

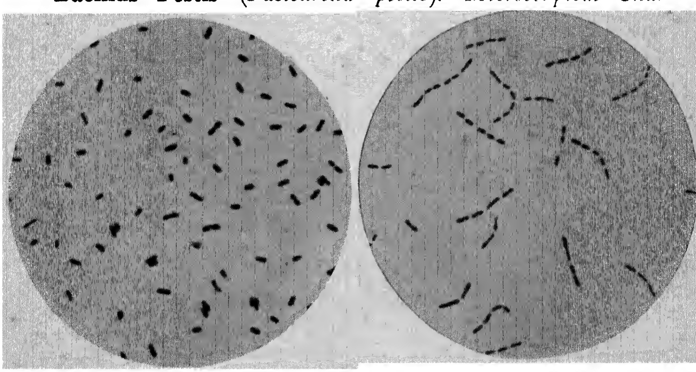


FIG. 129.—Bacillus of plague from a young culture on agar. Stained with weak carbol-fuchsin. ×1000.

FIG. 130.—Bacillus of plague in chains, showing polar staining. From a young culture in broth. Stained with carbol-thionin. ×1000.

acters.—As seen in the affected glands or buboes in this disease, the bacilli are small oval rods, somewhat shorter than the typhoid bacillus, and about the same thickness (Fig. 128), averaging 1·5 by 0·7 μ, though considerable variations in size occur. They have rounded ends, and in stained preparations a portion in the middle of the bacillus is often left uncoloured, giving the so-called "polar staining." In films from the tissues they are found scattered amongst the cells, for the most part lying singly, though pairs are also seen. On the other hand, in fluid cultures, *e.g.* broth, they grow mainly in chains, sometimes of considerable length, the form known as streptobacillus resulting (Fig. 130). In young agar cultures the bacilli show greater variation in size, and polar staining is less marked than in the tissues (Fig. 129); sometimes forms of considerable

length are present. After a time involution forms appear, especially when the surface of the agar is dry ; but the formation of these is much more rapid and more marked when 2 to 5 per cent. of sodium chloride is added to the medium, constituting the so-called " salt agar " (Hankin and Leumann). On this medium the involution forms assume a great size and a striking variety of shapes, large globular, oval, or pyriform bodies like yeasts resulting (Fig. 131). Sometimes in the tissues they are seen to be surrounded by an unstained capsule, though this appearance is by no means common. By means of India ink preparations, a capsule, in the form of a rather ill-defined envelope, has also been observed in cultures grown at 37° C. though it may be absent at lower temperatures. The bacilli do not form spores, and are non-motile. They stain readily with the basic aniline stains, and are Gram-negative.

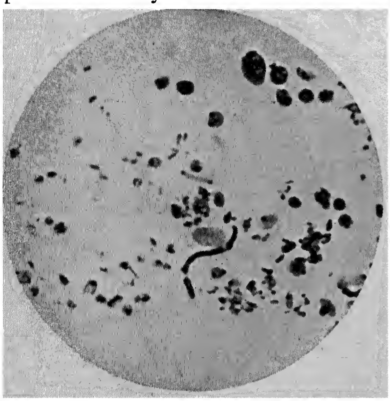


FIG. 131.—Culture of the bacillus of plague on 4 per cent. salt agar, showing involution forms of great variety of size and shape. See also Plate IV., Fig. 18.
Stained with carbol-thionin. ×1000.

Cultivation. — From the affected glands, etc., the bacillus can readily be cultivated on the ordinary media. It grows at the temperature of the body, though the optimum temperature of freshly isolated strains is lower, about 27° C., and growth occurs even at 14° C. In general the organism grows rather slowly in cultures. On *agar* and on *blood serum* the colonies are whitish circular discs of somewhat transparent appearance, and with a smooth, shining surface. When examined with a lens, their borders appear slightly wavy. After several days' incubation the colonies become larger, reaching 3 to 4 mm. in diameter, and develop a raised opaque centre with a thin transparent border. Growths sometimes have a viscous consistence. When agar cultures are kept at room temperature some of the colonies may show a luxuriant growth with more opaque appearance than the rest of the culture, the appearance in fact being often such as to suggest the presence of a mixed growth. Individual

colonies may thus vary considerably in character, but such variations do not as a rule constitute stable differences. In stab cultures in *gelatin*, growth takes place along the needle track as a white line, composed of small spherical colonies. On the surface of the gelatin a thin, semi-transparent layer may form, which is usually restricted to the region of puncture, though sometimes it may spread to the wall of the tube. There is no liquefaction of the medium. In *broth* the growth usually forms a slightly granular or powdery deposit at the foot and sides of the container, somewhat resembling that of a streptococcus. If oil or melted butter is added to the broth so that drops float on the surface, then a striking form of growth may result, to which the term " stalactite " has been applied. This consists in the growth starting from the under surface of the fat globules and extending downwards in the form of pendulous, string-like masses. These masses are exceedingly delicate, and readily break off on the slightest shaking; accordingly during their formation the culture must be kept absolutely at rest. This manner of growth constitutes an important but not absolutely specific character of the organism; unfortunately it is not exhibited by all strains of the organism, and varies from time to time with the same strain.

The organism grows under both aerobic and anaerobic conditions. Attention has been drawn to the sensitiveness of *B. pestis* to atmospheric oxygen and the difficulty of initiating growth when only a small number of organisms are inoculated on plates of culture medium; this difficulty is obviated if blood or sodium sulphite is added or if air is excluded (Schütze and Hassanein). Wright has pointed out that the organism may be killed by exposure to more than 1 per cent. of oxygen at a temperature of 37° C. *B. pestis* invariably ferments glucose and laevulose and usually galactose, mannitol, maltose, and salicin, in all cases without gas production. Lactose, saccharose, and dulcitol are not acted on. It does not form indole. Growth is not inhibited on a bile-salt medium. Nitrite is produced. Hydrogen sulphide is not formed.

The organism in its powers of resistance corresponds with other spore-free bacilli, and is readily killed by heat, an exposure for an hour at 58° C. being lethal. On the other hand, it has remarkable powers of resistance against cold; it has been exposed to a temperature several degrees below freezing-point without being killed. Experiments on the effects of drying have given somewhat diverse results, but as a rule the organism has been found to be dead after being dried in air for one or two days,

though sometimes it has survived the process for a longer period; exposure to direct sunlight for three or four hours kills it. The general result has been to show that the organism does not remain alive in natural conditions for long outside the animal body. In cultures, virulence may be retained for long periods.

Pathological Effects and Distribution of Bacilli.—The disease occurs in several forms, the *bubonic* and the *pulmonary* being the best recognised; to these may be added the *septicæmic*. The most striking feature in the *bubonic* form is the affection of the lymphatic glands, which undergo intense inflammatory swelling, attended with hæmorrhage, and generally ending in a greater or less degree of necrotic softening if the patient lives long enough. The connective tissue around the glands is similarly affected. The bubo is thus usually formed by a collection of enlarged glands fused by the inflammatory swelling. True suppuration is rare. Usually one group of glands is affected first, constituting the primary bubo—in the great majority the inguinal or the axillary glands—and afterwards other groups may become involved, though to a much less extent. Along with these changes there is great swelling of the spleen, and often intense cloudy swelling of the kidneys, liver, and other organs. There may also occur secondary areas of hæmorrhage and necrosis, chiefly in the lungs, liver, and spleen, and occasionally in the skin. Pneumonia may occur as a complication. The bacilli occur in enormous numbers in the swollen glands, being often so numerous that a film preparation made from a scraping almost resembles a culture (Fig. 128). In sections of the glands in the earlier stages, the bacilli form dense masses in the lymph paths and sinuses (Fig. 132); they may also be seen growing as a fine reticulum between the cells of the lymphoid tissue. At a later period, when disorganisation of the gland has occurred, they become irregularly mixed with the cellular elements. Later still they gradually disappear, and when necrosis is well advanced it may be impossible to find any—a point of importance in connection with diagnosis. In the spleen they may be very numerous or they may be scanty, according to the amount of blood infection which has occurred; in the secondary lesions mentioned they are often abundant. In the *pulmonary* form the lesion is the well-recognised "plague pneumonia." This is of broncho-pneumonic type, though large areas may be formed by confluence of the consolidated patches, and the inflammatory process is usually attended by much hæmorrhage; the bronchial glands show inflammatory swelling. Clinically there is usually

a fairly abundant frothy sputum often tinted with blood, and in it the bacilli may be found in large numbers. Sometimes, however, cough and expectoration may be absent. The disease in this form is almost invariably fatal; it is also extremely contagious. In the *septicæmic* form proper there is no primary bubo discoverable, though there is almost always slight general enlargement of lymphatic glands; here also the disease is of

FIG. 132.—Section of a human lymphatic gland in plague, showing the injection of the lymph paths and sinuses with masses of plague bacilli—seen as black areas.
Stained with carbol-thionin. ×50.

specially grave character. A bubonic case may, however, terminate with septicæmia; in fact, all intermediate forms occur. In the various forms of the disease the bacilli occur also in the blood, in which they may be occasionally found during life by microscopic examination, chiefly, however, just before death in very severe and rapidly fatal cases. The examination of the blood by means of blood-culture is, however, a much more reliable procedure. It may be said from the results of different

investigators that the bacillus may be isolated in fully 50 per cent. of the cases, though the number will necessarily vary in different epidemics. The Advisory Committee appointed by the Secretary of State for India in 1905, found that in some septicæmic cases the bacilli may be present in the blood in large numbers two, or even three, days before death, though this is exceptional.

The above types of the disease are usually classified together under the heading *pestis major,* but there also occur mild forms to which the term *pestis minor* is applied. In these latter there may be a moderate degree of swelling of a group of glands, attended with some pyrexia and general malaise, or there may be little more than slight discomfort. Between such and the graver types, cases of all degrees of severity are met with.

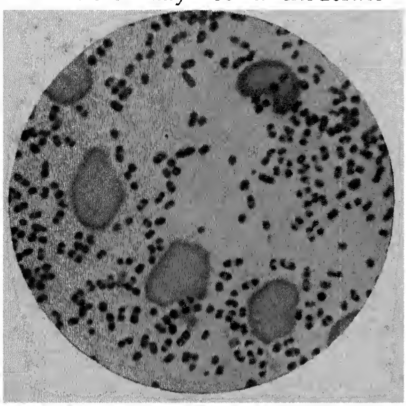


FIG. 133.—Film preparation of spleen of rat after inoculation with the bacillus of plague, showing numerous organisms, most of which are somewhat plump. Stained with carbol-thionin. ×1000.

Experimental Inoculation. — Guinea-pigs, mice, rats, and rabbits are susceptible to inoculation, the first being on the whole most suitable for experimental purposes. After subcutaneous injection there occurs a local inflammatory œdema, which is followed by inflammatory swelling of the corresponding lymphatic glands, and thereafter by a general infection. The lesions in the lymphatic glands correspond in their main characters with those in the human subject, although usually at the time of death they have not reached a stage so advanced. By this method of inoculation mice usually die in one to three days, guinea-pigs and rats in two to five days, and rabbits in four to seven days. *Post mortem* the main changes, in addition to the glandular enlargement, are congestion of internal organs, sometimes with hæmorrhages, and enlargement of the spleen; the bacilli are numerous in the lymphatic glands and usually in the spleen (Fig. 133), and also, though in somewhat less

degree, throughout the blood. Infection can also be produced by smearing the material on the conjunctiva or mucous membrane of the nose, or on an area of shaved skin, and this method of inoculation has been successfully applied in cases where the plague bacilli are present along with other virulent organisms, *e.g.* in sputum along with pneumococci. Rats and mice can also be infected by feeding either with pure cultures or with pieces of organs from cases of the disease, though in this case infection probably takes place through the mucous membrane of the mouth and adjacent parts, and only to a limited extent, if at all, by the alimentary canal. Monkeys also are highly susceptible to infection, and it has been shown in the case of these animals that when inoculation is made on the skin surface, for example, by means of a spine charged with the bacillus, the glands in relation to the part may show the characteristic lesion and a fatal result may follow without there being any noticeable lesion at the primary seat. This fact throws important light on infection through the skin in the human subject. Organisms of diminished virulence may produce chronic infections on inoculation, *e.g.* in rats.

Paths and Modes of Infection.—Plague bacilli may enter the system by the skin surface through small wounds, abrasions, etc., and in such cases there is usually little or no reaction at the site of entrance. This last fact is in accordance with what has been stated above with regard to experiments on monkeys. The path of infection is shown by the primary buboes, which are usually in the glands through which the skin is drained, those in the groin being the commonest site. Absolute proof of the possibility of infection by the skin is supplied by several cases in which the disease has been acquired at post-mortem examinations ; in the majority of these the lesions of the skin surface were of trifling nature, and there was no local reaction at the site of inoculation. The ordinary mode of skin infection is by means of the bites of fleas transmitting the bacilli from infected rodents, *e.g.* rats, as has been established by the work of the Advisory Committee referred to above. It had previously been shown that when fleas were allowed to feed on animals suffering from plague, plague bacilli might be found for some time afterwards in the stomach, and some observers, for example Simond, had succeeded in transmitting the disease to other animals by means of the infected insects. Most observers, however, had obtained negative results, but the Committee showed by carefully planned experiments that the disease could be transmitted from a plague rat to a healthy rat, kept in

adjacent cages, when fleas were present; whereas this did not occur when means were taken to prevent the access of fleas, though the facilities for aerial infection were the same. The disease can also be produced by fleas removed from plague rats and transferred directly to healthy animals, success having been obtained in fully 50 per cent. of experiments of this kind. When plague-infected guinea-pigs are placed among healthy guinea-pigs, comparatively few of the latter acquire the disease if fleas are absent or scanty; whereas all of them may die of plague when fleas are numerous. This result demonstrates the comparatively small part played by direct contact, even when of a close character. Important results were also obtained with regard to the mode of infection in houses where there had been cases of plague. It was found possible to produce the disease in susceptible animals by means of fleas taken from rats in plague houses. When animals were placed in plague houses and efficiently protected from fleas they remained healthy; whereas they acquired the disease when the cages were free to the access of fleas in the neighbourhood.

The following are some of the experiments which were conducted: A series of six huts were built which only differed in the structure of their roofs. In two the roofs were made of ordinary native tiles in which rats freely lodge; in two others, flat tiles were used in which rats live, but in which they have not such facilities for movement as in the first set, and in the third pair the roof was formed of corrugated iron. Under the roof in each case was placed a wire diaphragm which prevented rats or their droppings having access to the hut, but which would not prevent fleas falling down on to the floor of the hut. The huts were left a sufficient time to become infested with rats, and then on the floor in each case healthy guinea-pigs mixed with guinea-pigs artificially infected with plague were allowed to run about together. In the first two sets of huts to which fleas had access the healthy guinea-pigs contracted plague, while in the third set they remained unaffected, though they were freely liable to contamination by contact with the bodies and excreta of the diseased animals. In the third set of huts no infection took place as long as fleas were excluded, but when accidentally these insects obtained admission, then infection of the uninoculated animals commenced. Other experiments were also performed. In one case healthy guinea-pigs were suspended in a cage 2 inches above a floor on which infected and flea-infested animals were running about. Infection of the animals in the cage occurred; but if the cage were suspended at a distance above the floor higher than a flea could jump, then no infection took place. Again, in a hut in which guinea-pigs had died of plague, and which contained infected fleas, two cages were placed, each containing a monkey. One cage was surrounded by a zone of sticky material broader than the jump of a flea, another was left without this protection. The monkey in the former cage remained unaffected, but the other monkey contracted plague.

Other experiments showed that when plague bacilli were placed on the floors of houses, they died in a comparatively short period of time. After forty-eight hours it was not found possible to reproduce plague by inoculation with material from floors which had been grossly contaminated with cultures of the bacillus. Afterwards, however, animals placed in such a house might become infected by means of fleas. In all these experiments the common rat-flea of India—*Xenopsylla cheopis*—was used, but it has been shown that this flea also infests and bites the human subject. Recent observations show that not only is plague transferable by means of fleas, but that this is practically the only method obtaining in natural conditions, with the exception that rats may become infected by eating the carcases of other animals containing large numbers of plague bacilli. It is improbable from the experiments made that bubonic plague is transmitted by direct contact even when of a close nature; in fact, it has been shown that plague-infected guinea-pigs may suckle their young without the latter acquiring the disease. *The general results show that in the bubonic type fleas in nearly all cases are the vectors of infection.* In addition to *X. cheopis* certain other species of rat-fleas may also transmit the infection to man, e.g. *Ceratophyllus fasciatus*.

The later work of the Committee supplied information of the highest value with regard to the epidemiology of the disease; it showed, in short, that plague in its epidemic form is dependent on the epizootic among rats, and with regard to this some further facts may be given. Plague in Bombay occurs in two chief species of rats, the *Rattus rattus*, the black house-rat, and *Rattus norvegicus* (*decumanus*), the common brown rat of the sewers. The former, owing to its presence in dwelling-houses, is chiefly responsible for the transmission of the disease to man; while the latter, on account of the large number of fleas which infest it, is of special importance in maintaining the disease from season to season. The year may be divided into two portions—an epizootic season, from December to May inclusive, and a non-epizootic, from June to November. During the latter period there are few cases of plague in rats on account of fleas being scanty; especially is this so in the case of *Rattus rattus.* In fact, in certain villages where this species alone is present, the disease may actually die out at the end of the epizootic season, and accordingly when plague reappears in these places this is due to a fresh importation—a fact of great practical importance. A fresh epizootic first affects chiefly *Rattus norvegicus*, and a little later spreads to *Rattus rattus*, while

a little later still the disease attacks the human subject in the epidemic form; in each case fleas form the vehicle of transmission, and an interval of from ten to fourteen days intervenes between the outbreak of the epizootic and that of the epidemic. The proportion of cases of plague in *Rattus norvegicus* is much higher than in *Rattus rattus*, for the reason mentioned. It has been shown that a large proportion of the fleas removed from plague-infected rats contain plague bacilli, and that the fleas may remain infective for a considerable number of days, sometimes for a fortnight. After a flea has sucked infected blood from an animal the bacilli multiply in the stomach and proventriculus so that the latter structure becomes more or less blocked by the growth of bacilli. When this insect bites a new host, blood is sucked into the œsophagus and may in part enter the proventriculus or stomach, but later it regurgitates into the bite wound after becoming contaminated with bacilli. In this way a heavy inoculation results (Bacot and Martin). The subsidence of plague when the mean temperature rises above a certain level (about 80° F.) is probably in part, at least, due to the fact that the bacilli disappear much more rapidly from the alimentary tract of fleas at the higher temperatures; in accordance with this, experimental transmission of the disease to animals by means of fleas is more frequently successful at lower temperatures, and it has been shown that a temperature about 50° F. and an atmospheric humidity approaching saturation constitute the most favourable conditions for the survival of the organisms in the insect.

As regards the dying out of epidemics, some interesting facts have been brought forward by Liston. He and his co-workers have shown that rats taken from different towns vary greatly in their susceptibility to inoculation with plague bacilli, and that immunity is most marked in the rats from the towns which have suffered most severely from plague. This relative immunity appears to be due to the survival of the more resistant animals, and holds also with regard to their young. The diminution of plague amongst rats, and thus the subsidence of an epidemic, accordingly depends on the killing off of the more susceptible animals.

Enzootic and epizootic plague may occur in other wild rodent animals, *e.g.* the ground-squirrel in California, the gerbille and multimammate mouse in South Africa, the tarabagan or Siberian marmot in Manchuria, the spermophiles and field-mice in south-west Russia; and the infection in these animals, as in rats, may give rise to human cases or outbreaks.

In primary plague pneumonia, from a consideration of the anatomical changes and the clinical facts, the disease may be said to be produced by the direct passage of the bacilli into the respiratory passages by inhalation. The sputum droplets sprayed into the air by infected persons in the act of coughing carry the plague bacilli, which may survive for some time in cold wet weather, when the humidity of the atmosphere is high. In a dry atmosphere they die out quickly (Teague and Barber). Accordingly a case of plague pneumonia may be of great infectivity in producing other cases of plague pneumonia. Small epidemics of plague pneumonia break out from time to time, but in 1911 an extensive epidemic occurred in Manchuria leading to 50,000 deaths in six months. In this epidemic, direct infection from patient to patient was clearly shown, and rats were not concerned in the spread. Plague pneumonia appears to occur first of all as a complication in a bubonic case, and there is no evidence that the bacilli differ in virulence in the two conditions.

Toxins, Immunity, etc.—As is the case with most organisms which extensively invade the tissues, the toxins in plague cultures are chiefly contained in the bodies of the bacteria. Injection of dead cultures in animals produces distinctly toxic effects ; *post mortem*, hæmorrhage in the mucous membrane of the stomach, areas of necrosis in the liver, and at the site of inoculation, may be present. The toxic substances are comparatively resistant to heat, being unaffected by an exposure to 65° C. for an hour. By the injection of dead cultures in suitable doses, a certain degree of immunity against the living virulent bacilli is obtained, and, as first shown by Yersin, Calmette, and Borrel, the serum of such immunised animals confers a degree of protection on small animals such as mice. On these facts the principles of preventive inoculation and serum treatment, presently to be described, depend. It may also be mentioned that the filtrate of a plague culture possesses a very slight toxic action, and the Indian Plague Commission found that such a filtrate has practically no effect in the direction of conferring immunity.

Preventive Inoculation—Haffkine's Method.—To prepare the vaccine, cultures are made in flasks of broth with drops of oil on the surface (in India Haffkine employed a medium prepared by digesting goat's flesh with hydrochloric acid at 140° C. and afterwards neutralising with caustic soda). In such cultures stalactite growths (*vide supra*) form, and the flasks are shaken every few days so as to break up the stalactites and induce fresh crops. The flasks are kept at a temperature of about 25° C., and growth is allowed to proceed for about six weeks. Sterilisation

has usually been effected by exposing the contents of the flasks to 65° C. for an hour (*vide infra*); thereafter carbolic acid is added in the proportion of 0·5 per cent. The contents are well shaken to diffuse thoroughly the sediment in the fluid, and are then distributed in small sterilised bottles for use. The vaccine thus contains both the dead bodies of the bacilli and any toxins which may be in solution. It is administered by subcutaneous injection in the dose prescribed. Usually only one injection is made, sometimes two, though the latter procedure does not appear to have any advantage. The method has been systematically tested by inoculating a certain proportion of the inhabitants of districts exposed to infection, leaving others uninoculated, and then observing the proportion of cases of disease and the mortality among the two classes. The results of inoculation have been distinctly satisfactory. For although absolute protection is not afforded by inoculation, both the proportion of cases of plague and the percentage mortality among these cases have been considerably smaller in the inoculated as compared with the uninoculated. Protection is not established till some days after inoculation, and lasts for a considerable number of weeks, possibly for several months (Bannerman). In the Punjab during the season 1902–03 the case incidence among the inoculated was 1·8 per cent., among the uninoculated 7·7 per cent., while the case mortality was 23·9 and 60·1 per cent. respectively in the two classes, the statistics being taken from villages where 10 per cent. of the population and upwards had been inoculated.

According to statistics recently collated by Taylor in India, vaccination with Haffkine's prophylactic yields roughly a fourfold protection against attacks and an eightfold protection against death.

Plague vaccines have also been prepared from cultures on solid medium in accordance with the general methods used for vaccine preparation. In the past immunisation experiments in animals have indicated that the use of living non-virulent cultures as vaccine produces a higher degree of immunity than results from injecting killed organisms. This type of vaccine has been used with apparent success in the Dutch East Indies (Vogel). Sokhey and Maurice have recently pointed out the importance of using a fully virulent strain in preparing the Haffkine vaccine. They have shown that the original practice of killing the organisms at 60° to 70° C. injures the immunising properties of the vaccine, and an exposure at 55° C. for fifteen minutes is preferred. The final preparation is standardised by protection tests in white mice, and apparently a heat

killed vaccine from a virulent strain is more effective in such animal tests than a vaccine consisting of a non-virulent living culture.

Antigenic Structure.—It has been shown by Schütze that the plague bacillus possesses two types of antigen. One of these, which is thermolabile at 100° C., is contained in the envelope or capsule, while the other is thermostable and somatic, pertaining to the body of the organism. The envelope and the surface antigen are only fully formed when the organism is growing at 37° C. and cultures grown at lower temperatures, *e.g.* 25° C., as in the Haffkine vaccine, may lack this antigen. Schütze has pointed out that vaccines prepared from cultures grown at the higher temperature possess greater immunising properties in virtue of the envelope antigen which they contain, and his work offers a criticism of the standard method which has been used for preparing the plague vaccine in India. He has also correlated the protective value of anti-plague sera with their content of antibody for the envelope antigen. As mentioned above, this antigen is sensitive to heat and its preservation in a heat-sterilised vaccine requires careful consideration. Heating at 100° C. for fifteen minutes may render the vaccine useless; the antigen is also damaged by heating at 70° C. Thirty minutes exposure at 56° C. before addition of phenol does not, however, affect the immunising properties of the vaccine. No essential antigenic differences exist among strains of *B. pestis*. According to Schutze this organism exists only in the "rough" form. Its antigenic relationships to other members of the *Pasteurella* group will be dealt with later.

Anti-plague Sera.—Of these, two have been used as therapeutic agents, namely, that of Yersin and that of Lustig. Yersin's serum is prepared by injections of increasing doses of plague bacilli into a horse. In the early stages of immunisation dead bacilli are injected subcutaneously, thereafter into the veins, and, finally, living bacilli are injected intravenously. After a suitable time blood is drawn off and the serum is preserved in the usual way. Of this serum 10 to 20 c.c. are used, and injections are usually repeated on subsequent days. Lustig's serum is prepared by injecting a horse with repeated and increasing doses of a substance derived from the bodies of plague bacilli, probably in great part nucleo-protein. Masses of growth are obtained from the surface of agar cultures, and are broken up and dissolved in a 1 per cent. solution of caustic potash. The solution is then made slightly acid by hydrochloric acid, when a bulky precipitate forms; this is collected on a filter and dried. For use, a weighed amount is dissolved in a weak solution of carbonate of soda and then injected. The serum is obtained from the animal in the usual way. Extensive observations with both of these sera show that neither of them can be considered a powerful remedy in cases of plague, though in certain instances distinctly favourable results have been recorded. The Indian Commission, however, came to the conclusion "that, on the whole, a certain amount of advantage accrued to the patients in cases both of those injected with Yersin's serum and of those injected with Lustig's serum." It may also be mentioned that the Commission found, as the result of experiments, that Yersin's

serum modified favourably the course of the disease in animals, whereas Lustig's serum had no such effect. By immunising cattle with a virulent strain of *B. pestis* a highly potent anti-serum, as judged by experimental tests, has been obtained (Naidu, F. P. Mackie and Brist).

Plague bacteriophage.—D'Herelle has recorded favourable therapeutic results from the injection of a bacteriophage for *B. pestis* into the buboes, but the efficacy of this procedure has not been confirmed by others. The question has also been investigated experimentally in plague-infected animals, but the results do not indicate that such bacteriophage has a definite curative value (Pirie, Compton.)

Serum Diagnosis.—Specific agglutinins may appear in the blood of patients suffering from plague, as also they do in the case of animals immunised against the plague bacillus. It is to be noted, however, that in clinical cases the reaction is not invariably present, the potency of the serum is not of high order, and the carrying out of the test is complicated by the natural tendency of the bacilli to cohere in clumps. For the last reason the macroscopic (sedimentation) method is to be preferred to the microscopic (p. 139). A suspension of plague bacilli is made by breaking up a young agar culture in 0·85 per cent. sodium chloride solution ; the larger flocculi of growth are allowed to settle, and the fine, supernatant emulsion is employed in the usual way. According to the results of the German Plague Commission and the observations of Cairns, made during the Glasgow epidemic, it may be said that the reaction is best obtained with dilutions of the serum of from 1 : 10 to 1 : 50. Cairns found that the date of its appearance is about a week after the onset of illness, and that it usually increases till about the end of the sixth week, thereafter fading off. It is most marked in severe cases characterised by an early and favourable crisis, less marked in severe cases ultimately proving fatal, while in very mild cases it is feeble or may be absent. The method, if carefully applied, may be of service under certain conditions ; but it will be seen that its use as a means of diagnosis is restricted. The use of high-titre agglutinating antisera obtained from horses is of value for identifying culture of *B. pestis* ; there is, however, agglutinating action also on *B. pseudotuberculosis rodentium*.

Methods of Diagnosis.—Where a bubo is present a little of the "juice" may be obtained by puncture and aspiration with a sterile hypodermic syringe. The fluid is then to be examined microscopically, and cultures on agar or serum-agar should be made by the successive stroke method. The morphological, cultural and biochemical characters are investigated. The pathogenic properties should also be studied , the guinea-pig being on the whole most suitable for subcutaneous inoculation. In many cases a diagnosis may be made by microscopic examination alone, as in no known condition other than plague do bacilli with the morphological characters of the plague bacillus occur in large numbers in the lymphatic glands. The organism may be obtained in culture from the blood in a considerable proportion of cases by withdrawing a few cubic centimetres and proceeding in the usual manner. On the occurrence of the first suspected case, every care to exclude possibility of doubt should be used before a positive opinion is given.

In a case of suspected plague pneumonia, in addition to micro-

scopic examination of the sputum, the above cultural methods and animal inoculation with the sputum should be carried out; smearing the nasal mucous membrane or a freshly shaved area of skin of a guinea-pig may be recommended. Here a positive diagnosis should not be attempted by microscopic examination alone, especially in a plague-free district, as bacilli morphologically resembling the plague organism may occur in the sputum in other conditions.

Recognition of Plague Infection in Rats and other Wild Rodents.—This is of great importance in plague prevention. In the autopsy of the suspected rat careful attention is first paid to the various groups of lymph glands, particularly those of the neck which are most frequently affected. The glands show marked enlargement with surrounding œdema and often hæmorrhages, and plague bacilli may be demonstrated in smears from the tissue. The spleen is enlarged and the liver shows often a characteristic mottling due to yellow necrotic areas. There is usually marked congestion of the subcutaneous vessels, often with hæmorrhages. Effusion into the pleuræ may be noted. In an animal recently dead after an acute infection the plague bacilli may be demonstrated in the heart blood and spleen. It often happens, however, that carcases of rats found dead and requiring examination for plague infection are decomposed, and it may be impossible to demonstrate the bacilli microscopically and isolate them by the usual methods of culture. The existence of the infection may often be established in such cases by inoculating a shaved and scarified area of skin in a guinea-pig with material from the lesions. The plague bacilli invade the tissues and produce a typical plague infection. All the characters of a suspicious organism must be investigated since recently isolated strains of coliform bacilli on subcutaneous inoculation in guinea pigs may cause lesions closely resembling those due to *B. pestis* (Levinthal) and in rats other *Pasteurella* organisms may have a similar action. It should be noted that the typical lesions are not necessarily the same in different species of rodents; for example, in the guinea pig the spleen shows areas of focal necrosis, which are not common in the rat.

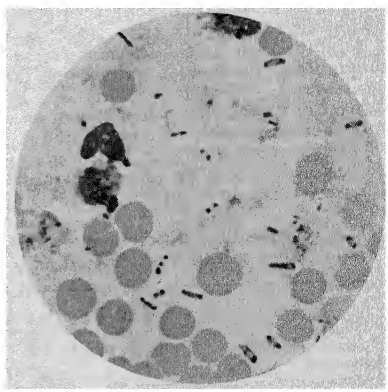


FIG. 134.—Film preparation from lung of rabbit, dead of Pasteurella infection. ×1000. (Leishman's stain.)

Pasteurella Group.—It is noteworthy in reference to the bacteriology of plague, that the *B. pestis* is a member of a group of bacteria (designated the *Pasteurella* group) which are responsible for epizootic disease among various animal species. Organisms of this group are causally associated with the condition of hæmorrhagic

septicæmia among fowls (*B. avisepticus*), rabbits (*B. lepisepticus*) (Fig. 134), cattle (*B. bovisepticus*), pigs (*B. suisepticus*), etc. Though they have been separately named according to the animals in which they occur, it is doubtful if they should be regarded as different species or types. These organisms all present morphological and cultural characters similar to those of the plague bacillus. In the investigation of plague among animals, the differentiation of *B. pestis* from other organisms of the group may be of considerable importance. It was shown by the Indian Plague Commission that the hæmorrhagic septicæmia organisms are incapable of growing in media containing sodium taurocholate (*e.g.* MacConkey's medium), while *B. pestis* can be grown on this medium. These organisms may also be differentiated from *B. pestis* by their fermentation of saccharose and production of indole. An investigation of the pathogenicity and effects of strains in question under experimental conditions in different laboratory animals should also be carried out, as well as the agglutination reaction with a potent antiserum.

An organism of the *Pasteurella* group also occurs in a pseudo-tuberculous disease of guinea-pigs and other rodents (*B. pseudo-tuberculosis rodentium*). This species differs from *B. pestis* in respect of its motility when grown at room temperature (Arkwright) and shows one or two flagella at one or both ends. Some strains also ferment saccharose. Cultures in milk become alkaline, whereas *B. pestis* produces no change in milk. An important criterion in differentiating it from *B. pestis* is its lack of virulence for the white rat.

The outstanding differences among the organisms of the *Pasteurella* group can be summarised as follows :

	B. pestis.	*B. avisepticus* (exemplifying the hæmorrhagic septicæmia organisms).	*B. pseudo-tuberculosis rodentium.*
Motility at 22° C.	−	−	+
Formation of acid from saccharose	−	+	±
Action on milk	−	−	alkalinised
Indole production	−	+	−
Inhibition of growth by bile-salt	−	+	−
Virulence for white rat	+	+	−

B. pestis when first isolated can be differentiated from *B. pseudo-tuberculosis rodentium* by making dilutions of cultures, adding small inocula to rabbit-blood-agar and incubating at 37° C.; *B. pseudo-tuberculosis rodentium* grows well under these conditions within twenty-four hours whereas *B. pestis* only shows small colonies after forty-eight hours. The formation of nitrite (*vide* p. 511) is also said to distinguish *B. pestis* from *B. pseudotuberculosis rodentium* which does not exhibit this property.

The antigenic relationships of the *Pasteurella* group are of particular interest. Serologically, all these organisms show some degree

of relationship, and that between *B. pestis* and *B. pseudotuberculosis rodentium* has been fairly well defined. Reference has been made above to the double antigenic structure of the plague bacillus and it has been suggested that this organism exists only in the " rough " form. According to Schütze serological types of *B. pseudotuberculosis rodentium* can be differentiated and this organism may occur in the " smooth " or the " rough " phase. When cultivated at 22° C. it possesses a thermolabile flagellar antigen which is common to the different types. A thermostable somatic and type-specific antigen is characteristic of the " smooth " phase, while another somatic antigen, in this case common to the species, can be identified in the " rough " form. This " rough " somatic antigen is also the same as the somatic component of *B. pestis*. The hæmorrhagic septicæmia organisms, though possibly sharing antigenic components with the other members of the *Pasteurella* group, can usually be distinguished from these by quantitative agglutination tests. As to the serological relationships or differences among the hæmorrhagic septicæmia strains it is difficult at present to draw any definite conclusions from the various observations.

TULARÆMIA (DEER-FLY FEVER, OR OHARA'S DISEASE)

Bacterium Tularense.—This organism was first described by McCoy and Chapin in a plague-like disease of ground-squirrels in California. The infection also occurs in other wild rodents, *e.g.* rabbits, and has been found in sheep and birds. The organism is a very minute bacilloid or coccoid body measuring 0·3 μ to 0·7 μ in its longest diameter. It stains Gram-negatively and in films from animal lesions often presents the appearance of a capsule. It can be demonstrated microscopically in the spleen of infected animals where it may be present in enormous numbers. Artificial cultures were first obtained on coagulated egg-yolk (6 parts of fresh hen-egg yolk and 4 parts of saline solidified by heating at 72° C. for thirty minutes and sterilised by heating again at 73° C. for one hour). Francis has devised a medium containing meat extract, 1 per cent. peptone, 1·5 per cent. agar and 0·5 per cent. NaCl, adjusted to a pH of 7·3; before use 0·1 per cent. cystine and 1 per cent glucose are added, and the mixture is steamed for fifteen minutes in the Koch, then cooled to 45° to 50° C. and 5 to 10 per cent. rabbit blood or 5 per cent. sterile human serum added. Growth may also be obtained on blood-agar and serum-glucose-agar containing a piece of sterile rabbit's spleen.

The infection, spoken of as tularæmia, is transmissible to man, and a considerable number of cases have now been recorded in North America and also in Norway, Russia, and Japan. The disease is characterised by an indolent primary lesion on the skin, with persistent enlargement of the related lymph-glands, which tend to break down, as well as constitutional disturbance; but fever may be the only symptom. In fatal cases pneumonic patches are present in the lung in about 70 per cent. The exudate is usually mononuclear and necrosis is common. Similar focal lesions in the liver are found in about half of the cases. Certain of the cases have resulted from contact with infected animals, *e.g.* through handling diseased rabbits and other rodents. Cases may arise also from the bite of blood-sucking flies and ticks, and strains

of *Bact. tularense* have been isolated from ticks. It is specially remarkable how readily laboratory workers may become infected by handling infective material and artificial cultures, and cases of such laboratory infection have occurred both in America and in this country, leading to a somewhat protracted period of invalidism. Artificial cultures apparently retain a remarkably high degree of virulence and infectivity. Laboratory animals can be infected experimentally from cultures, and at autopsy the principal pathological changes are multiple necrotic foci in the spleen, liver, and lymph nodes The pathological picture varies considerably in different organs and in different species According to Ledingham, the spleens of infected guinea-pigs dead of tularæmia may show characteristic lesions along with diffuse polymorphonuclear leucocyte infiltration. In such specimens the organisms cannot be demonstrated microscopically. On the other hand, in mice the spleen may show a much lesser degree of pathological change with no leucocytic response and films from the tissue contain large numbers of organisms Ledingham has also noted that in mice the cells of the liver parenchyma are invaded by the organism in large numbers, and the cell cytoplasm is often replaced by them. It has been found that the infection may be passed experimentally to laboratory animals by infected blood-sucking insects.

In human cases the serum agglutinates *Bact tularense* in dilutions up to 1 : 1200 or higher, the maximum titre being reached after four to seven weeks ; thereafter it falls, but persists for years above that of persons who have never been infected.

It is of special interest that this organism shows serological relationships to the *B melitensis-abortus* group (Francis and Evans) and has recently been classified in the *Brucella* group, though originally placed in the *Pasteurella* group.

CHAPTER XIX

THE ORGANISMS OF UNDULANT FEVER AND EPIZOOTIC ABORTION (BRUCELLA GROUP)

Bacillus Melitensis and Malta Fever

Malta or undulant fever due to *B. melitensis* is of common occurrence along the shores of the Mediterranean and in its islands. Although from its symptomatology and pathological anatomy it has been recognised as a distinct affection, and was known under various names, its precise etiology was unknown till the publication of the researches of Bruce in 1887. From the spleen of patients dead of the disease he cultivated the organism, now known as the *Bacillus* (or *Brucella*) *melitensis*, and by means of inoculation experiments established its causal relationship. Wright and Semple applied the agglutination test to the diagnosis of the disease, and in 1904 its mode of spread was fully studied by a Commission, whose work demonstrated that goat's milk is the chief source of infection. Since its bacteriology has been worked out, it has been found to occur also in India, China, South Africa, and in some parts of North and South America, its distribution being much wider than was formerly supposed.

The duration of the disease is usually long—often two or three months, though shorter and much longer periods are met with. Its course is very variable, the fever being of the continued type with irregular remissions. In addition to the usual symptoms of pyrexia, there occur profuse perspiration, pains and sometimes swellings in the joints, occasionally orchitis, while constipation is usually a marked feature. The mortality is low—about 2 per cent. (Bruce).

In fatal cases the most striking post-mortem change is in the spleen. This organ is enlarged, often weighing slightly over a pound, and in a condition of acute congestion ; the pulp is soft and may be diffluent, and the Malpighian bodies are swollen and indistinct. In the other organs the chief change is cloudy swelling ; in the kidneys there may be in addition glomerular

nephritis. The lymphoid tissue of the intestines shows none of the changes characteristic of typhoid fever.

It is now recognised that an undulant fever resembling the classical Malta fever may also be due to *Bacillus abortus*, the organism of epizootic abortion of cattle and certain other domestic animals, which is closely related to *Bacillus melitensis*. Human infections by *Bacillus abortus* have a wide geographical distribution corresponding with that of the infection in animals.

Bacillus Melitensis (*Brucella melitensis*).—This is a small, rounded, oval or cocco-bacillary organism about 0·4 μ in diameter, which is specially abundant in the spleen. It usually occurs singly or in pairs, but in cultures short chains are also met with (Fig. 135). The coccal form of the organism originally led to its being designated a micrococcus. In culture, however, its bacillary character is more obvious, and rod-shaped forms about 0·6 to 1·5 μ in length may be observed. It stains fairly readily with the ordinary basic aniline stains. In Gram's method it reacts negatively. It is non-motile. In the spleen of a patient dead of the disease it occurs irregularly scattered through the congested pulp; it may also be found in small numbers *post mortem* in the capillaries of various organs. It may be cultivated from the blood during life in a considerable proportion of cases. The organism was found by the members of the Commission in the urine of Malta fever patients in 10 per cent. of the cases examined; it was sometimes scanty, but sometimes present in large numbers. It has also occasionally been obtained from the fæces.

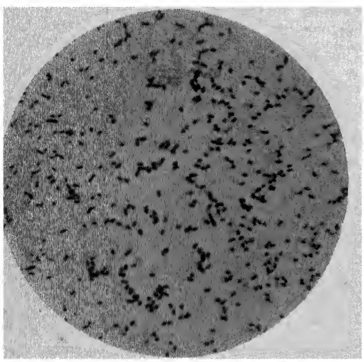


FIG. 135.—*Bacillus melitensis*, from a two days' culture on agar at 37° C. Stained with dilute carbol-fuchsin. × 1000.

Cultivation.—Growth occurs under aerobic conditions on ordinary media, *e.g.* agar, at 37° C., but for isolation and routine cultivation, a liver-extract-agar gives the best results. In primary culture, the colonies, which develop slowly, appear as small round discs, slightly raised and of somewhat transparent

appearance. The maximum size—2 to 3 mm. in diameter—is reached about the ninth day; at this period by reflected light they appear pearly white, while by transmitted light they have a yellowish tint in the centre, bluish-white at the periphery. After being accustomed to artificial medium the organism grows more abundantly and more rapidly. The optimum temperature is 37° C., but growth still occurs down to about 20° C. In gelatin at 22° C. growth is extremely slow—after two or three weeks, in a stab culture, there is a delicate line of growth along the needle track and a small flat expansion of growth on the surface. There is no liquefaction of the medium. In broth there occurs a general turbidity with flocculent deposit at the bottom. The reaction of media should be about pH 6·6 to 6·8. On potato the growth assumes after several days' incubation a characteristic chocolate-brown colour. Growth on various sugar media fails to reveal any obvious fermentative properties, but it has been shown that utilisation of glucose occurs in culture. Outside the body the organism has considerable powers of vitality, as it has been found to survive in a dry condition in dust and clothing for a period of two months. The thermal death point is about 60° C. Reference will be made later to the differentiation of *B. melitensis* from *B. abortus*.

Relations to the Disease.—There is, in the first place, ample evidence from examination of the spleen, both *post mortem* and during life, that this organism is always present in the disease. The experiments of Bruce and Hughes first showed that by inoculation with even comparatively small doses of pure cultures the disease could be produced in monkeys, sometimes with a fatal result. And it has now been fully established that inoculation with the minutest amount of culture, even by scarification, leads to infection both in monkeys and in the human subject.

Rabbits, guinea-pigs, and mice are relatively insusceptible to inoculation by the ordinary method. Durham, by using intracerebral inoculation, however, succeeded in raising the virulence, so that the organism was capable of producing illness in guinea-pigs on intraperitoneal injection with sometimes a fatal result many weeks afterwards. Eyre also, after increasing the virulence by intracerebral inoculation, was able to produce active infection in various animals, especially on intravenous injection. Intramuscular injection of large doses of culture in guinea-pigs may set up infection, but the disease is usually retrogressive. If the animal is killed after six to eight

weeks following inoculation, necrotic lesions may be observed in the spleen and liver, and sometimes also in other tissues, and from these the organism can be cultivated.

Mode of Spread of the Disease.—The work of the 1904 Commission resulted in establishing facts of the highest importance with regard to the spread of the disease. In the course of investigations Zammitt found that the blood serum of many of the goats agglutinated the *Bacillus melitensis*, and Horrocks obtained cultures of the organism from the milk. Further observations showed that the agglutination reaction occurred in 50 per cent. of the goats in Malta, while the organism was present in the milk in 10 per cent. Sometimes it was present in enormous numbers, and in these cases the animal usually appeared poorly nourished, and the milk had a somewhat serous character. In other cases, however, it was present when the animals appeared healthy, and there was no physical or chemical change discoverable in the milk. It was also determined that the organism might be excreted for a period of two to three months before any notable alteration occurred in the milk. The milk of infected animals usually agglutinated the bacillus, and this property was always present when the bacillus was found in the milk. It may be noted here that while these carrier animals show little disturbance of health, abortion may be associated with the infection especially in flocks lacking the protection of an inherited immunity. It was found by the Commission that monkeys and goats could be readily infected on feeding them with milk containing the organism, the disease being contracted by fully 80 per cent. of the monkeys used. It was therefore rendered practically certain that the human subject was infected by means of such milk, and the result of preventive measures, by which milk was excluded as an article of dietary amongst the troops in Malta, fully bore out this view. After such measures were instituted, the number of cases in the second half of 1906 fell to 11 per thousand, as contrasted with 47 per thousand in the corresponding part of the preceding year ; cases now are relatively few. Various facts with regard to the epidemiology of the disease were thus cleared up. For example, it was more prevalent in the summer months, when more milk was consumed ; and there was a larger proportion of cases among those in good social position, the officers, for example, suffering more in proportion than the rank and file. Another interesting fact, pointed out by Horrocks, is that the disease practically disappeared from Gibraltar after the practice of importing goats from Malta was stopped.

The work of the Commission excluded other modes of infection than the ingestion of infected milk as being of practical importance; if the disease is conveyed by contact at all, this is only when the contact is of an intimate character, and even then it is probably of rare occurrence.

There is distinct evidence that the disease may be acquired by inoculation through small lesions in the skin, and this method is probably not infrequent among those who handle infected milk. It has been shown that the organism may remain alive in the bodies of mosquitoes for four or five days, and it might seem theoretically possible that insects may occasionally be the means of carrying the disease; there is no evidence, however, that this takes place.

It has also been found that sheep may suffer under natural conditions from infection by *B. melitensis*.

Reference is made later, under *B. abortus*, to the question of the relationship of this organism to undulant fever in the human subject.

Agglutination Reaction.—The blood serum of patients suffering from Malta fever possesses the power of agglutinating the *Bacillus melitensis* in a manner analogous to what has been described in the case of typhoid fever. The reaction appears comparatively early, often about the fifth day, and may be present for a considerable time after recovery—sometimes for more than a year. The serum of apparently normal persons may agglutinate *B. melitensis* occasionally in relatively high dilution, *e.g.* 1 : 50 and sometimes even 1 : 100. In the disease, however, the agglutination reaction usually occurs with much higher dilutions, *e.g.* 1 : 500, or over.

The Commission found that vaccination with dead cultures of the organism confers a certain degree of protection among those exposed to the disease. As a rule, two injections were given, 200–300 million organisms being the dose of the first injection, and about 400 million the dose of the second. The use of vaccines has also been carried out in the treatment of the disease, but the results are not sufficiently conclusive to establish its value.

Methods of Diagnosis.—During life the readiest means of diagnosis is supplied by the agglutination test (for technique, *vide* p. 138).

Cultures may sometimes be obtained from the blood by the usual methods. Cultures are most easily obtained from the spleen either during life by spleen puncture or *post mortem*. Plates of liver-extract agar are inoculated and incubated aerobically at 37° C. Film preparations should also be made from the spleen pulp and stained with carbol-thionin and by Gram's method.

In the identification of the organism agglutination by an antiserum to a known strain would supply confirmatory proof, as in the methods used for the typhoid-paratyphoid group (*vide* p. 521). It is to be noted that strains which are serologically different in direct agglutination tests from the common type of *B. melitensis* have been met with and the name *B. para-melitensis* has been given to these. It is supposed that such strains represent a "rough" variant of *B. melitensis*.

Great care must be exercised in working with cultures of the *B. melitensis*, as bacteriologists have become infected apparently from such sources in an unusually high proportion of instances.

The serological relationship of *B. melitensis* and *B. abortus* is referred to later and also the differentiation of these organisms by cultural and other methods (*vide infra*).

Bacillus Abortus

Bacillus abortus (*Brucella abortus*) presents close biological similarity to the *B. melitensis*. It is important in human medicine on account of its frequent occurrence in cow's milk, and the possibility of its causing undulant fever in the human subject. The organism was first described by Bang in 1897 as the cause of bovine infectious abortion. The infection has a wide geographical distribution and has been long prevalent in milch cows, among which at the present time in this country it is exceedingly common. The infection is not limited to cattle, but occurs in pigs, *e.g.* in the United States. *B. abortus* has been found occasionally in horses and certain other mammalian animals, and also in fowls. In morphology *B. abortus* is a very small, pleomorphic, non-motile, Gram-negative bacillus usually not exceeding 1 to 1·5 μ in length and 0·3 to 0·8 μ in breadth, and often coccal in form like *B. melitensis*. Longer forms up to 3 μ may sometimes be observed on a rich medium. From material in which the organism is likely to be present in a state of purity, *e.g.* the heart blood of the fœtus, it may be cultivated, according to Bang's original method, in the form of a shake-culture in a tube of serum agar. In primary growth the colonies develop after inoculation in a zone about 10–20 millimetres below the surface of the medium. This behaviour was taken as evidence that the organism was micro-aerophilic (p. 18); recent work of Wilson and others has indicated, however, that growth is not favoured by lowering the oxygen-pressure below that of the air. Smith had shown previously that it will grow readily in first culture in an atmosphere containing up to 10 per cent. of carbon dioxide, and the presence of 5–10 per cent. of this gas along with 20 per cent. oxygen appears to afford the optimum atmosphere for cultivation of most strains of *B. abortus*. Growth occurs in sealed tubes, apparently because carbon dioxide

is given off by the organisms and by various culture media and its retention in the tube favours growth (Wilson). The explanation of the characteristic zonal growth, which has been described above, seems to be that in a tube of medium in which a shake-culture has been made any carbon dioxide given off at the surface is lost and growth does not occur, while in the depth of the medium anaerobic conditions restrain growth, but where the carbon dioxide and oxygen are maintained at a suitable partial pressure growth results. It is unknown how carbon dioxide influences growth in this way ; but the effect is not apparently due to change in the hydrogen-ion concentration of the medium. After continued cultivation the organism can be grown under ordinary conditions, the colonies being small transparent discs resembling those of *B. melitensis*. On potato *B. abortus* produces a brownish or chocolate-coloured growth similar to that of *B. melitensis*. It has no obvious fermentative action on carbohydrates. In addition to the so-called *bovine type* of *B. abortus* found in cattle, another variety occurs in swine and is designated the *porcine type* (or *Brucella suis*). This type, like *B. melitensis*, does not exhibit the carbon dioxide requirement for growth of the bovine type and can grow under ordinary aerobic conditions. Both types produce hydrogen sulphide for the first four days in a medium containing organic sulphur (p. 82) ,whereas *B. melitensis* forms none. The porcine type is the more active in this respect. The selective action of the dyes thionin and basic fuchsin, incorporated in plates of culture medium, has also been utilised for differentiating the three types. The following table summarises the differences :

	Carbon dioxide requirement.	Hydrogen sulphide production.	Inhibition of growth by	
			Thionin 1 : 50,000.	Basic fuchsin 1 : 50,00.
B. melitensis .	—	—	—	—
B. abortus, bovine .	+	+	Inhibition	—
B. abortus, porcine (American type) . .	—	++	—	Inhibition

Methyl violet (1:50,000) and pyronin (1:100,000) yield results similar to those with basic fuchsin. Porcine strains of *B. abortus* occurring in Denmark differ from the American variety in failing to produce hydrogen sulphide and in being more susceptible to inhibition by dyes.

That *B. abortus* is responsible for bovine abortion has been proved by experimental infection in pregnant cows, *e.g.* by injection of cultures into the vagina or by feeding. Intravenous injection in pregnant rabbits and guinea-pigs may also lead to abortion in these animals. The infection in cattle is usually associated with little general effects. The development of the fœtus is arrested, and there may be inflammation of the fœtal membranes. The organism may persist in the genital organs for some time after the abortion ; it localises in the udder and is discharged in the milk for a varying period so that the animal becomes a carrier of the infection. The agglutination reaction of the serum with known cultures is applied in the recognition of infected animals, and even low end-titres, *e.g.* 1 in 20 are regarded as significant.

The bovine type of *B. abortus* produces an active but non-fatal infection in guinea-pigs associated with necrotic lesions in lymph glands, spleen, and liver. The porcine type is of higher virulence to these animals and in monkeys produces a more severe infection than the bovine type.

The occurrence of *B. abortus* in undulant fever (" abortus fever ") of the human subject is now well recognised and cases have been recorded in various countries including Great Britain. Porcine strains are apparently of greater virulence to man than those of bovine origin. The infection may occur in persons whose occupation brings them into contact with diseased animals, *e.g.* abattoir workers, farmers, etc., and the organism can readily invade the body through abraded skin, but many cases of " abortus fever " are undoubtedly due to infection from cow's milk, and it has been clearly shown that *B. abortus* is present in a considerable proportion of samples of raw market milk : in Edinburgh, in 1932, 34·9 per cent. of a series of samples were found infected. It is, in fact, remarkable that the infection is not more prevalent in view of the occurrence of the organism in cow's milk. In this connection it may be noted that young subjects are relatively immune and in any case the average susceptibility of adults to the bovine strains must be comparatively low. The illness varies considerably in its general symptoms ; most cases due to the bovine strains are mild, and a proportion may be ambulatory. The fever is undulant in type and is associated with profuse night sweats and often pains in the joints ; arthritis may occur as a complication. In certain cases in which the disease has occurred in pregnant women abortion has resulted (Hasseltine). Morales-Otero records experiments in which human volunteers be-

came infected as the result of either drinking milk containing a strain of the organism on repeated occasions, or inoculating an area of abraded skin once with the organism ; positive results were got with *B. melitensis* and with both bovine and porcine types of *B. abortus*, by both routes in each case. The diagnosis rests on the results of bacteriological examination. Sometimes the organism may be isolated by blood-culture but this method often gives negative results even in undoubted cases. The agglutination reaction with the patient's serum and known strains of *B. abortus* generally offers a reliable means of diagnosis, and the titre is frequently high, *e.g.* 1 in 1000 to 1 in 6000 ; it must be remembered, however, that *B. abortus* may be agglutinated by the serum of apparently normal persons in a dilution of 1 : 50. It should be noted that persons may contract the infection without overt signs, and it has been suggested from the results of agglutination tests with sera submitted to laboratories for other examinations, that over one per cent. of the general community in this country are so infected.

As shown by Evans, *B. abortus* and *B. melitensis* are so closely related serologically that they cannot be differentiated by direct agglutination tests. Agglutinin-absorption tests, however, enable the two organisms to be separated. The study of a number of strains showed that the majority of those of bovine and porcine origin were serologically of the *B. abortus* type. One strain of *B. melitensis* type was also isolated from an aborted bovine fœtus and another from a goat after abortion. A *B. para-abortus* has been described which can be differentiated from *B. abortus* by direct agglutination tests. It is apparently analogous to *B. para-melitensis*. It has been suggested that *B. para-abortus* is a rough variant of the typical form. The "roughness" of strains can be ascertained by the thermo-agglutination test, *i.e.* boiling in saline for two hours.

The relationship of *B. melitensis* and the two types of *B. abortus* has been well illustrated in a study of their antigenic structure by Wilson and Miles. They have postulated two antigenic constituents (*A* and *M*). In the bovine type *A* predominates quantitatively ; in *B. melitensis, M* ; while the American porcine type is intermediate, *A* predominating but to a lesser degree than in the bovine variety. It seems likely from further studies of strains of these organisms that there are other intermediate types each possibly characteristic of a particular locality, some classified as *B. melitensis*, others as *B. abortus*. Thus a Rhodesian type derived from cattle and classified as *B. abortus* has certain characters in which it

resembles *B. melitensis* (*e.g.* ability to grow without carbon dioxide) and it is apparently of high virulence to the human subject.

Methods of Examination.—When primary cultures are required from material likely to contain other organisms, *e.g.* from the genital passages of infected cows, cultivation presents difficulty and requires special methods. Smith's method, *i.e.* growth in an atmosphere, 5 to 10 per cent. of which has been replaced by carbon dioxide, provides the most convenient means of obtaining primary cultures. The required concentration of carbon dioxide to yield surface growths in sub-cultures may be obtained by flaming the cotton-wool plug of the culture tube for two to three seconds, then pushing it in and fifteen seconds later closing the tube with an india-rubber stopper or sealing it (Wilson). A liver-extract agar (to which may be added gentian-violet 1 : 100,000) has been found most suitable. Cultures should be kept at 37° C. for seventy-two hours or longer. For the demonstration of *B. abortus* in milk, the subcutaneous inoculation of a guinea-pig may be resorted to; after four to eight weeks the animal is killed and the organisms can be recovered from the spleen in which they are present in considerable numbers. Direct cultures may also be made from the cream which separates after specimens have been kept for twenty-four hours in the ice-chest (Huddleson, Hasley and Torrey).

The agglutination test, and the diagnosis of undulant fever have been referred to earlier.

Other organisms biologically allied to B. melitensis are the *Bacillus of guinea-pig pneumonia*, described by Smith, and the *B. bronchisepticus* reported by Ferry and by McGowan as associated with canine distemper. This latter organism is not the specific virus of distemper but only a secondary invader. It is noteworthy that *B. bronchisepticus* differs from other members of the *Brucella* group in its motility and the possession of peritrichous flagella.

CHAPTER XX

THE TETANUS BACILLUS: OTHER PATHOGENIC ANAEROBIC BACILLI

Introductory.—Tetanus is a disease which in natural conditions affects chiefly man and the horse. Clinically it is characterised by the gradual onset of general stiffness and spasms of the voluntary muscles, commencing in those of the jaw—"lock-jaw"—and the back of the neck, and extending to all the muscles of the body. These spasms are of a tonic nature, and, as the disease advances, succeed each other with only a slight intermission of time. There are often, towards the end of a case, fever and rise of respiration and pulse-rate. The disease is usually associated with a wound received ordinarily from four to fourteen days previously, which has been contaminated by manured soil or excretal matter. The disease is, in the majority of cases, fatal.

Historical.—The general association of tetanus with the presence of wounds, though these might be very small, suggested that some infection took place through the latter, but for long nothing was known as to its nature. Carle and Rattone (1884) produced the disease in a number of animals by inoculation with material from a wound in tetanus. Nicolaier (1885) infected mice and rabbits with garden earth, and found that many of them developed tetanus. In these animals suppuration occurred in the neighbourhood of the inoculation, and on microscopic examination of the pus, besides other organisms, a characteristic bacillus was found. Inoculation of fresh animals with such pus reproduced the disease. Attempts at its isolation were, however, unsuccessful, although inoculation of animals with the mixed culture produced the disease. These results were confirmed by Rosenbach, who cultivated the other organisms present, and inoculated them, but with negative results. He further pointed out, as characteristic of the bacillus, its development of a terminal spore. In 1889, Kitasato succeeded in isolating from the local suppuration of mice inoculated from a human case a bacillus which when injected in pure culture into mice, rats, guinea-pigs and rabbits, subcutaneously or intravaneously, caused the disease, and which was named *B. tetani*. Kitasato found that the cause of earlier culture failures was the fact that it could only grow in the absence of oxygen. The pathology of the disease was further elucidated by Faber, who, having isolated bacterium-free poisons from cultures, reproduced the characteristic symptoms.

Bacillus Tetani

As seen in young cultures, *B. tetani* (*Clostridium tetani*) appears as a slender organism, usually about 4 μ to 5 μ in length and about 0·4 μ in thickness, with somewhat rounded ends. Besides occurring as shorter rods, it also develops filamentous forms, the latter being more common in fluid media. It stains readily and uniformly by any of the usual stains. It stains positively by Gram's method, but does not retain the stain strongly, since both in the tissues and in culture numerous Gram-negative individuals occur. It is

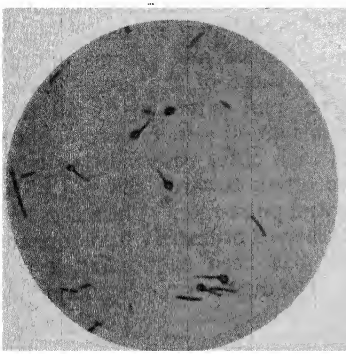


Fig. 136.—Tetanus bacilli; some of which possess spores. From a culture in glucose agar, incubated for three days at 37° C. See also Plate IV., Fig. 21.
Stained with carbol-fuchsin. ×1000.

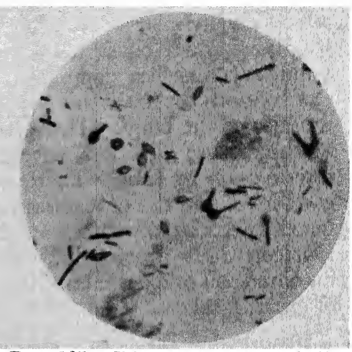


Fig. 137.—Film preparation of discharge from wound in a case of tetanus, showing several tetanus bacilli of "drum-stick" form. (The thicker bacillus present is not a tetanus bacillus, but a putrefactive anaerobe which was obtained in culture from the wound.)
Stained with gentian-violet. ×1000.

slightly motile, its motility being best studied in an anaerobic hanging-drop preparation. Numerous delicate peritrichous flagella can be demonstrated by special staining (Fig. 138); they may be of considerable length, but are usually curled up close to the body of the bacillus. As is the case with many other anaerobic flagellated bacteria, the flagella tend to become detached, and massed together in the form of spirals of striking appearance (Fig. 139). At incubator temperature *B. tetani* readily forms spores, which are terminal. They are at first oval, but when mature are round, and in diameter may be two or three times the thickness of the bacilli. Hence the spored bacillus presents the "drum-stick" form (Figs. 136, 137). In

a specimen stained with methylene-blue, the fully developed spores are uncoloured except at the periphery, so that the appearance of a small ring is produced; if a powerful stain such as carbol-fuchsin be applied for some time, the spores become deeply coloured like the bacilli. In the discharge from a wound infected with *B. tetani*, both spore-bearing and spore-free forms are usually to be found, the latter having no distinctive features.

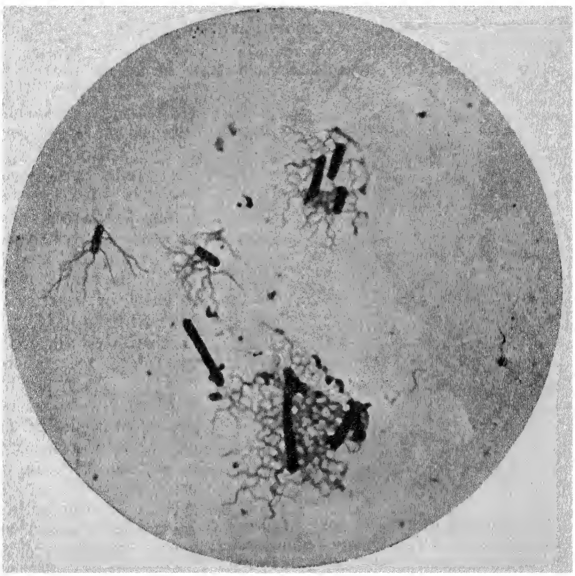


FIG. 138.—Tetanus bacilli, showing flagella. Stained by Rd. Muir's method. ×1000.

Occurrence in Nature.—By inoculation of animals, *B. tetani* has been proved to be frequent in richly manured soil, *e.g.* garden earth; it also occurs in street dust. It flourishes in the intestines of many—especially herbivorous—animals and such carriers are responsible for distributing the spores in nature. It has also been recovered from the intestine of man. In soil the organism occurs in the form of spores, and it is doubtful whether the tetanus bacillus can exist as a saprophyte. In turn spores from the soil are ingested by animals and germinate in the intestine. It is not definitely known whether the carrier state in animals is transient or permanent.

Isolation.—(1) The principle is to take advantage of the resistance of the spores of the bacillus to heat. A tube of cooked-meat medium (p. 59) is inoculated and incubated at 37° C. for forty-eight hours, at the end of which time spore-bearing bacilli can often be observed microscopically. The culture is then kept at 70° C. for from three-quarters to one hour, with a view to killing all organisms except those which have spored. From such material anaerobic plate cultures are prepared by the methods described on p. 92 *et seq.* This method of isolation is frequently unsuccessful, because along with the tetanus bacilli, both in its natural habitats outside the body and in the pus of wounds, other spore-forming obligatory and facultative anaerobes occur, which grow faster than the tetanus bacillus, and thus overgrow it.

(2) The method by which Fildes has isolated many strains depends upon the fact that under suitable conditions *B. tetani* grows as a spreading film which extends beyond the growth of other organisms. With material from a case of tetanus it is advisable to enrich the bacilli first by inoculating a tube of freshly boiled blood broth (p. 69), which is incubated anaerobically at 37° C for two to four days. Then from this culture the condensation water of a sloped tube of digest blood agar is inoculated and incubated anaerobically at 37° C. After twenty-four to forty-eight hours the extreme edge of the film of growth, seen with a hand-lens as a tangle of extremely fine filaments, is found on microscopic examination to consist of a culture of the tetanus bacillus; subcultures from the margin yield pure growths of the organism. The detection of the edge of the growth is facilitated by using agar tubes which have been kept until the medium has become slightly dry at the top. The only organism which is likely to spread as extensively as *B. tetani* is *Bacillus proteus*. The latter can be eliminated by heating the mixed growth containing spores of *B. tetani*.

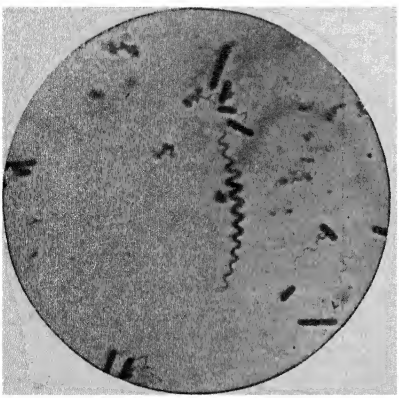


FIG. 139.—Spiral composed of numerous twisted flagella of the tetanus bacillus. Stained by Rd. Muir's method. ×1000.

Characters of Cultures and Conditions of Growth.—In the case of the ordinary media growth takes place only in the absence of free oxygen, the organism being an *anaerobe*; but when particulate matter is present, *e.g.* in cooked-meat medium, *B. tetani* like other anaerobes will grow without complete

exclusion of the air. It has been shown recently by O'Meara that if broth be used which is free from traces of copper, it is only necessary to heat it at 100° C. to expel dissolved oxygen and then on inoculation an abundant growth of *B. tetani* results. In deep *glucose gelatin* (in which growth is often very difficult to obtain) there commences, an inch or so below the surface, a growth consisting of fine straight threads, rather longer in the lower than in the upper parts of the tube, radiating out from the needle track (Fig. 140). Slow liquefaction of the gelatin takes place, with slight gas formation. In *agar* the culture is somewhat similar, there being a slightly nodular line of growth along the needle track, with irregular short offshoots passing out into the medium (Fig. 149, A) and slight formation of gas. On anaerobic agar plates colonies have under a low power a feathery outline (Fig. 141). On gelatin plates the colonies consist of a thick centre with shoots radiating out on all sides. In fluid media under anaerobic conditions there is at first a slight turbidity, and later a thin layer of a powdery deposit on the walls of the vessel. All the cultures have a peculiar odour of burnt organic matter. Meat medium undergoes no change in colour. None of the sugars commonly used are fermented. *Coagulated serum* is softened but not liquefied. On *blood agar* hæmolytic zones develop round the colonies. On making subcultures in fluid media a considerable amount of the original growth should be used for the inoculation.

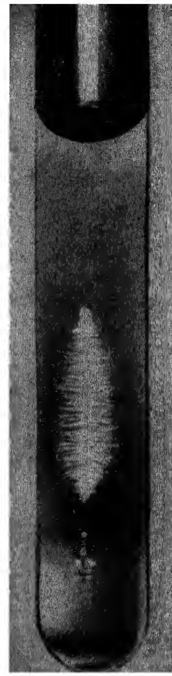


FIG. 140.—Stab culture of the tetanus bacillus in glucose gelatin, showing the lateral offshoots (after Kitasato).
Natural size.

B. tetani grows best at 37° C. The minimum growth temperature is about 14° C., and below 22° C. growth takes place very slowly. Sporulation may commence at the end of twenty-four hours in cultures grown at 37° C.—much later at lower temperatures. The spores may withstand boiling for forty to sixty minutes; but in some cases they may not survive for ten minutes. They can be kept in a dry condition for many months without being killed or losing their virulence. They have also high powers of resistance to

many antiseptics; for example, they withstand 5 per cent. phenol and 1 in 1000 mercuric chloride for two weeks or even longer. On the other hand, a 1 per cent. watery iodine solution or hydrogen peroxide (10 volumes) destroys them within a few hours.

Pathogenic Effects.—The proof that the *B. tetani* is the cause of tetanus is complete since pure cultures reproduce the disease. Failure to isolate it from some cases of the disease is very probably due to the small numbers in which it sometimes occurs. The tetanus bacillus by itself lacks the power of invading the tissues. It obtains a foothold only under favouring conditions such as are afforded by a mixed infection, the presence of foreign matter or necrotic tissue. That this is the case first appeared from experiments of Vaillard and others in which tetanus spores freed from toxin by heating at 80° C. were injected. The animals remained healthy, but the spores persisted in a living state for a considerable time, and tetanus was set up in guinea-pigs when, after some weeks, staphylococci were injected subcutaneously into the same site as the tetanus spores. Similarly in rabbits after intravenous injection of such detoxicated spores a simple fracture of the femur caused the disease to develop. Vaillard and his co-workers also noted that *B. prodigiosus*, various aerobic soil organisms, and mixtures of pyogenic organisms injected along with *B. tetani* spores led to the development of tetanus. The injection of sterile soil favours the germination of these spores in the tissues, and Bullock and Cramer have attributed this effect to the ionisable calcium salts in the soil. Various chemical substances (such as those capable of damaging or devitalising the tissues) have also been found to promote tetanus when injected along with spores, *e.g.* quinine, lactic acid. According to Tulloch, the toxin of *B. welchii* exerts a marked effect in predisposing to tetanus when *B. tetani* spores are injected. The factors that promote the development of *B. tetani* in the tissues have been studied by Mackie, McLachlan and Anderson, who found that certain

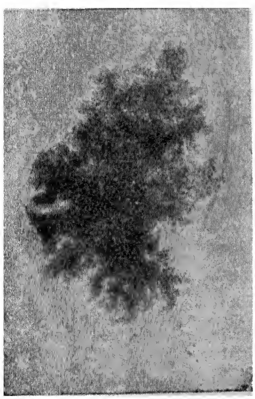


FIG. 141—Colony of the tetanus bacillus on anaerobic agar plate, seven days old. ×50.

animals, *e.g.* mice, can resist massive doses of spores *per se*; small doses, however, when injected along with sublethal doses of *B. welchii*, *B. œdematiens*, *B. coli*, and *Staphylococcus aureus*, frequently produced tetanus in these animals. Exposure to cold also precipitated tetanus in animals even a considerable time after injection of the spores. Vaillard and his co-workers attributed the non-pathogenicity of *B. tetani* spores to their being rapidly phagocytosed in the healthy tissues, and assumed that those factors which interfered with this protective mechanism, *e.g.* pyogenic infection, would favour their development in the body. Recently Fildes has claimed that the failure of the spores to germinate is due to the unfavourable oxygen tension of the healthy tissues, and that germination, with resultant tetanus, is dependent on factors producing tissue asphyxia. The inhibitory and adjuvant factors concerned are probably complex, and depend not only on phagocytosis and the oxygen tension of the tissues, but also on the parasitic properties of the organism and the various defences of the tissues. In experiments on tetanus following vaccination Armstrong found that when the site of inoculation with a mixture of vaccinia virus and tetanus spores was kept covered throughout by an impermeable dressing, the majority of the monkeys and rabbits so treated developed tetanus; but when the inoculated area was freely exposed to the air after the first eighteen hours, the occurrence of tetanus was exceptional. Tetanus bacilli tend to remain localised at the site of entry in a wound, and the toxin which they produce is absorbed and acts on the central nervous system.

(*a*) *The disease as arising naturally.*—It occurs chiefly in horses and in man. The spasms early affect the extensor muscles of the trunk (descending tetanus). There is in most cases a definite wound, often of a ragged character, which has either been made by an object soiled with earth or dung, or which has become contaminated with these substances. For example, tetanus has followed wounds received from toy pistols loaded with cartridges having contaminated wads. There is often a purulent or fœtid discharge, though this may be absent. It is important to note that the wound through which infection has taken place may be very small, in fact, may consist of a mere abrasion. In some cases, especially in the tropics, it may possibly be merely the bite of an insect. In some parts of the world infection through the umbilicus originates a high mortality from the disease in newly born infants. The absence in many cases of an obvious channel of infection has given rise to the

term "idiopathic" tetanus. In tetanus following clean operation wounds, imperfectly sterilised catgut ligatures have been the source of infection. Surgical catgut being prepared from the submucous layer of sheep's intestine, the raw material is liable to contamination with the spores of *B. tetani* and other intestinal anaerobes, e.g. *B. welchii,* and as a result of the spinning process these spores may come to be embedded in the ligature. The resistance of *B. tetani* spores to various physical and chemical agencies has been mentioned above, and in the past imperfectly sterilised catgut ligatures have not infrequently been used for surgical purposes.[1]

During the late war the clinical type of tetanus seen in the wounded was modified in consequence of the wide application of the prophylactic injection of anti-tetanic serum (*vide infra*). In the first place there was a tendency to the prolongation of the incubation period, instances where this was extended to many months being not uncommon. In such cases there was usually an unhealed septic wound, often containing foreign bodies, and the attack of tetanus was sometimes precipitated by operative procedures; in some cases the wound had healed and tetanus followed operation for the removal of foreign bodies in the tissues. Again the disease tended to assume the type seen in some animals, the muscles in the neighbourhood of the wound being first affected; local hardness and stiffness, pain, and exaggeration of local reflexes were often the first, and sometimes the only, clinical phenomena. Such cases of tetanus were also apparently more amenable to treatment with anti-tetanic serum.

The pathological changes found *post mortem* are not striking. There may be hæmorrhages in the muscles, which have been the subject of the spasms. These are probably due to mechanical causes. In the nervous system there is ordinarily a general redness of the grey matter, and the most striking feature is the occurrence of irregular patches of slight congestion which are not limited particularly to grey or white matter, nor to any tract of the latter.—These patches are usually most marked in the grey matter of the medulla and pons. Microscopically there is little of a definite nature to be found. There is congestion, and may be minute hæmorrhages in those areas red to the naked eye. The ganglion cells may show appearances which have been regarded as degenerative in nature, and similar changes have been described in the white matter. The only marked feature is thus a vascular disturbance in the central

[1] The sterility of surgical catgut is now controlled in this country under the provisions of the Therapeutic Substances Act.

nervous system, with a possible tendency to degeneration in its specialised cells. Both of these conditions are probably due to the action of the toxins of the bacillus. In the other organs of the body there are no constant changes.

(*b*) *The artificially produced disease.*—The disease can be communicated to animals by any of the usual methods of inoculation, but does not arise from feeding with the bacilli, whether these contain spores or not. In mice, after inoculation with a loopful of a broth culture, symptoms appear in a day, and death occurs in two or three days. Guinea-pigs and rabbits require larger doses, and death does not occur so rapidly. Usually in small animals injected subcutaneously in a limb the spasms begin in that limb near the point of inoculation and this is the rule when the inoculation is intramuscular (ascending tetanus). In the case of inoculation intravenously or into a non-muscular region, the spasms begin in the extensor muscles of the trunk, as in the natural disease in man (descending tetanus). After death there is found, at the seat of inoculation, slight hyperæmia without pus formation. The bacilli diminish in number, and may be absent at the time of death. The organs generally show little change.

Kitasato stated that in his earlier experiments the quantity of culture medium injected along with the bacilli already contained enough of the poisonous bodies formed by the bacilli to cause death. The symptoms came on sooner than by the improved method mentioned below, and were, therefore, due to the toxins already present. In his subsequent work, therefore, he employed splinters of wood soaked in cultures in which spores were present, and subsequently subjected for one hour to a temperature of 80° C. The latter treatment not only killed all the vegative forms of the organism, but was sufficient to destroy the activity of the toxins. When such splinters are introduced subcutaneously, death results by the development of the spores which they carry. In this way he completed the proof that the bacilli by themselves can form toxins in the body and produce the disease.

The Toxins of the Tetanus Bacillus.—The toxic properties of bacterium-free filtrates of pure cultures of the *B. tetani* were investigated in 1891 by Kitasato. When the filtrate, in suitable doses, was injected subcutaneously into mice, tetanic spasms developed first in muscles contiguous to the site of inoculation and later all over the body. Death resulted. He found that guinea-pigs were more susceptible than mice, and rabbits less so. The toxin is easily injured by heat; exposure

for a few minutes at 65° C. destroys it; it is also destroyed by twenty minutes' exposure at 60° C., and by one and a half hours' at 55° C. It is also destroyed by various chemicals such as pyrogallol and acids, and also by sunlight. Toxin is very stable when dried.

To prepare the toxin, freshly made veal broth not too long autoclaved should be used, and a massive inoculation, preferably from a fluid culture, practised. Individual strains of the bacillus differ in their capacity for producing toxin. The culture must be incubated under anaerobic conditions, and the maximum toxicity is developed in from five to fifteen days. Behring pointed out that after the filtration of cultures containing toxin, the latter may very rapidly lose its power, and in a few days may only possess 1/100th of its original toxicity. This is due to such factors as temperature and light, and especially to the action of oxygen. When dilutions of toxin are to be prepared, broth should be used and not saline, which leads to rapid deterioration. Toxin may be preserved by precipitation with ammonium sulphate; the precipitate is dried and kept as a powder. The effect of certain agents, *e.g.* formaldehyde, on the crude toxin is to form what it is convenient at present to call *toxoids*, similar to those produced in the case of diphtheria toxin; and it is also true here that the toxoids while losing their toxicity may still retain their power of producing immunity against the potent toxin. Further, altogether apart from the occurrence side by side in the crude toxin of strong and weak poisons, it has been shown that such crude toxin may contain different varieties of toxic substances. Ehrlich showed that beside the predominant spasm-producing toxin (called by him *tetanospasmin*), there often exists in crude toxin a poison capable of producing the solution of certain red blood corpuscles. This hæmolytic agent he called *tetanolysin*. It does not occur in all samples of crude tetanus toxin, nor is it found when a broth culture of the bacillus is filtered through porcelain. Tetanolysin also has the power of originating an antitoxin, so that certain anti-tetanic sera can protect red blood corpuscles against its action; this protective effect can occur even when the corpuscles have already combined with the lysin. Madsen, studying the interactions of this anti-tetanolysin with the tetanolysin, showed that phenomena can be demonstrated similar to those noted by Ehrlich as occurring with diphtheria toxin, which the latter interpreted as indicating the presence of degenerated toxins (toxoids) in the crude poison. A leucocidin has also been observed. It is important to note that the toxin may not be found in mixed cultures of *B. tetani* along with other organisms (Tulloch).

As with other members of the group, nothing is known of the nature of tetanus toxin. Uschinsky has found that the tetanus bacillus can produce its toxin when growing in a fluid containing no protein matter.

The toxin is one of the most powerful poisons known. Even with an impure "toxalbumin" consisting practically of an alcoholic precipitate from filtered cultures in broth, Brieger and Cohn found that the fatal dose for a mouse was 0·00005 of a milligram. If the susceptibility of man be the same as that of a mouse, the fatal dose

for an average adult would have been 0·23 of a milligram Animals differ very much in their susceptibilities to the action of tetanus toxin According to Fildes' compilation, if the minimal lethal dose per gram weight for a horse be taken as unity, that for the guinea-pig would be 2 times the amount, the mouse 4, the goat 6, the rabbit 24, the dog about 300, the cat 2400, and, according to v. Lingelsheim, the goose, 12,000, the pigeon 48,000, and the hen 360,000. These figures, however, may be affected by various factors such as the individuality of the animals, their age (the younger being more sensitive), etc While in a number of animal-species the lethal dose of toxin is practically the same on intravenous, intramuscular, or subcutaneous administration (van Genderen , Abel), on the other hand, different species vary in their sensitiveness to intracerebral injection as compared with the above methods. Thus the fatal dose of toxin for the guinea-pig is not smaller when it is injected intracerebrally than when given subcutaneously. In the rabbit, however, a much smaller dose of tetanus toxin proves fatal when injected into the brain than when given subcutaneously. This probably depends on the relative affinities of different organs for the toxin

Non-toxic Variants of B. tetani.—Fildes has shown that from typical toxigenic strains of *B. tetani* non-toxic variants are developed; and non-toxic forms identified as tetanus bacilli by serological criteria have been isolated by various workers from different sources, though they are apparently most frequent in soil

Tetanus toxin is readily absorbed and produces its characteristic effects when injected subcutaneously. intramuscularly, intravenously, intraperitoneally, or intrathecally. But it is not absorbed when placed on the surface of granulation tissue, *e.g.* in a wound a week old. That the toxin is not destroyed. however, is shown by the fact that if later the granulation tissue is broken down mechanically, absorption occurs and tetanus develops. In the healthy alimentary tract toxin does not lead to tetanus ; this is probably to be accounted for by non-absorption as well as by its destruction. In human cases it has occasionally been demonstrated in the blood and the cerebrospinal fluid.

A striking feature of the action of tetanus toxin is the occurrence of an incubation period between its introduction into an animal's body and the appearance of symptoms. This varies according to the species of animal employed ; on subcutaneous injection in the guinea-pig it is from thirteen to eighteen hours and in the horse five days. Increase of the dose, however, within limits shortens the incubation. In man the interval between the injury and the appearance of tetanic symptoms is usually from two to fourteen days, but this period may be lengthened, and the bacilli may remain a considerable time shut up in a wound before producing effects. The longer the

incubation period, the more favourable the prognosis, and in chronic cases spontaneous recovery is not uncommon.

With regard to the action of the toxin, it has been shown that when a minimum lethal dose is introduced directly into the blood-stream, it disappears rapidly from the circulation, or when injected subcutaneously it cannot be demonstrated in the blood at all. This, no doubt, is partly due to its passing into the lymph, but mainly to its combining with certain tissues, especially the central nervous system, and the latter has been found to fix the toxin *in vitro*. But when a large amount of the toxin is injected into the body or produced there, it circulates in the blood stream. Accordingly, it is a point of practical importance always to test antisera before their administration to the human subject, in order to ensure that they are free from tetanus toxin. Neglect of this precaution has led to fatalities from the employment of the serum of horses withdrawn during the incubation period of tetanus. The toxin has an effect on the central nervous system which can be clearly demonstrated by injecting it directly into the spinal cord. After an incubation period which is much shorter than when the toxin is injected by any other route, tetanic spasms start in the muscles corresponding to the site of inoculation and then spread to other muscles as the toxin diffuses in the cord. The spasms are due to the raised excitability to afferent stimuli which is produced by the toxin in the motor cells of the anterior cornula. According to the earlier investigators (Marie and Morax; Meyer and Ransom; Permin and others), the local muscular spasm is wholly explicable on this basis, the toxin reaching the cord by passing up the motor nerves. The precise mode of spread of toxin in the nerves remains doubtful, however. According to one view, the axis cylinders are the conductors; on the other hand, it has been held that the toxin ascends in the neural lymphatics or tissue spaces, but for these two routes there appears to be no anatomical evidence. According to the recent work of Abel and others, the local muscular contraction, which is the initial phenomenon in ascending tetanus and which also follows the direct injection of a minute dose of toxin into the substance of a muscle, is due to the local action of the toxin. Abel states that the injection of a considerable dose of tetanus toxin into the sciatic nerve of a dog is not followed by tetanus, either general or local, provided that leakage of the toxin into the surrounding tissues is prevented. But the occurrence of local spasm depends in some way on the intact condition of the connection of the muscle with the spinal cord by means of the

motor nerve. When the nerve is cut, a subsequent injection of toxin into the corresponding muscle does not produce spasm. On the other hand, when spasm has become fully developed, section of the motor nerve no longer abolishes it. In the light of this work the general muscular spasm of descending tetanus is accounted for by the diffuse action on the central nervous system of toxin conveyed to it by the circulation. In addition to the action on the cord, the toxin affects the brain, which probably explains the general convulsions also characteristic of the disease. This has been most clearly shown by injecting toxin into the vitreous humour of rabbits which had previously received an intravenous injection of antitoxin. The animals developed general convulsions with practically no tonic spasm (Permin). According to Sherrington's investigations, disturbance of reciprocal innervation also occurs from the action of the toxin on the brain. Thus, stimulation of an area of the cerebral cortex which in a healthy animal causes opening of the mouth—due to contraction of the depressors of the jaw accompanied by relaxation of the elevators—in a tetanic animal leads to trismus, since the normal inhibition of the closing muscles which form the more powerful group, is now converted into stimulation. An injection of toxin into the posterior nerve routes between the ganglion and the spinal cord does not produce tetanus, but causes severe spasmodic pains (*tetanus dolorosus*). Tetanus toxin, in addition to acting on the central nervous system, may injure other organs such as the liver, which has been found affected in animals dying in the course of active immunisation against the toxin. Some such general metabolic damage would explain also the intense wasting which occurs in subacute cases of tetanus intoxication in man and animals in spite of free intake of food.

Immunity against Tetanus.—Anti-Tetanic Serum.—It was in the case of tetanus toxin that antitoxin immunity was first demonstrated by Behring and Kitasato, who found that a degree of immunity could be conferred by the injection of very small and progressively increasing doses of the tetanus toxin. Subsequent work has shown that the richer a crude toxin is in modifications of the true toxin (toxoids), the more useful it is for immunisation procedures. In fact it is doubtful if small animals can be immunised at all by fresh filtrates. In some cases the injection of non-lethal doses instead of commencing an immunity actually increases the susceptibility of the animal, and this may be related to the development of supersensitiveness to proteins generally (see "Anaphylaxis," p. 260). Accordingly, toxin

modified by the action of iodine trichloride was originally used. It is found that the greater the degree of the natural susceptibility of an animal to tetanus, the easier is it to obtain a serum of a high anti-tetanic potency. The horse is, therefore, the most suitable animal and is usually employed. While tetanus antitoxin is frequently present naturally in the blood of cattle, sheep, and goats, it is absent from man, horses, pigs, and dogs.

For the immunisation of horses toxin-antitoxin mixtures or formol-toxoid may be injected to begin with and then, after an interval, toxin (p. **634**). The serum of such immune animals possesses the capacity of protecting animals susceptible to the disease against a subsequent injection of a fatal dose of tetanus bacilli or toxin Further, if injected subsequently to infection, the serum can in certain cases prevent a fatal result, even when symptoms have begun to appear The degree of success attained depends, however, on the shortness of the time which has elapsed between the injection of the bacilli or of toxin and the injection of the serum. In animals where symptoms have fully manifested themselves only a small proportion of cases can be saved As with other antitoxins, there is no evidence that the anti-tetanic serum has any detrimental effect on the bacilli. It only neutralises the effects of the toxin.

The standardisation of the anti-tetanic serum is of the highest importance. Behring originally recommended that a serum should be obtained of which 1 gram will protect one million grams weight of mice against the minimum fatal dose of the bacillus or toxin. The method now used is similar to that for diphtheria antitoxin (see Chapter VI.), either guinea-pigs or mice being used; and a standard dried toxin as well as antiserum are preserved for testing purposes The mixture of toxin and antitoxin is injected subcutaneously, and either the greatest amount of the latter is determined which fails to prevent death in four days, or, preferably, the least amount which prevents the appearance of tetanus. The strength of the antitoxin is reckoned in terms of similar units In this country the unit now used is the International Unit (I.U.) adopted by the League of Nations; this unit is half of that determined by the method practised in the U.S A

Alum-toxoid has been used for the production of active immunity in the human subject.

Agglutination.—Cultures of tetanus bacilli which have been washed and heated to deprive them of toxin, when injected into rabbits, lead to the production of agglutinins. By this means Tulloch has shown that the organisms fall into five main serological groups. It is to be noted, however, that there is no difference in the toxins of the different groups, as an antitoxin developed by one type neutralises the toxin from other types. Serological differences among strains depend apparently on the H or flagellar antigen.

The Therapeutic Application of Tetanus Antitoxin.—As the result of his experiments, Behring aimed at obtaining a

curative effect in the natural disease occurring in man by the use of antitoxic serum derived from large animals. The essential factors for the success of serum therapy are, first, that there should not be the least unnecessary delay in commencing treatment, and secondly, that the antitoxin should be given in proper amount. This has been demonstrated clearly by experiment. MacConkey determined the amount of antitoxin sufficient to save the life of a guinea-pig when injected under the skin simultaneously, but at another site, with a dose of toxin just sufficient to kill an untreated animal; he then found that one-tenth of this amount of serum sufficed to neutralise 100 fatal doses of toxin when toxin and antitoxin were mixed together *in vitro* prior to injection; and two thousand times as much serum was required to save the life of the animal when injected twenty-four hours after the toxin, *i.e.* at a time when symptoms of tetanus had already appeared. As has been seen, the toxin rapidly disappears from the blood stream; but even after this has occurred, antitoxin is still up to a point capable of exerting a curative effect. Whether the antitoxin can act by causing dissociation of toxin already fixed to vulnerable cells is, however, doubtful. The serum may be given subcutaneously, intramuscularly, intravenously, or intrathecally by lumbar puncture. In the first two methods absorption is relatively slow—in the last elimination is relatively rapid. But by the intrathecal method the serum gains rapid access to the grey matter of the cord on which the toxin is exerting its specific action. Sherrington treated monkeys inoculated with tetanus toxin, after the development of symptoms, either by an intrathecal or an intramuscular injection of antitoxin and showed the great superiority of the former method under the conditions studied. The earlier injections ought therefore to be given both intrathecally and also intravenously, the latter route being chosen because antitoxin can be quickly administered, and as Henderson Smith has shown, a high concentration of antitoxin in the body fluids is maintained for a considerable time; the neutralisation of toxin passing out from a focus of infection is thus facilitated. Later, injections should be given by intramuscular and subcutaneous routes, the principle being that, as the antitoxin first given is eliminated, its place is taken by the more slowly absorbed and therefore more gradually eliminated moieties. Andrewes has recommended the initial injection intrathecally of 20,000 to 30,000 U.S.A. units, *i.e.* 40,000 to 60,000 I.U. (in the form of concentrated antitoxin), and repeated doses may be given for several days;

20,000 to 30,000 I.U. are given intravenously along with the first intrathecal dose, and a similar amount is injected subcutaneously three or four days later. Yodh has obtained good results from combined intracisternal, intravenous, and intramuscular administration of the antiserum.

The results of the therapeutic use of antitoxin in tetanus have not been so good as in the corresponding case of diphtheria. The great difficulty is that an infection is not suspected till the tetanus toxin has already begun to manifest its gravest effects, by which time the nerve cells may have been damaged irreparably. In this connection the experimental results of Roux and Borrel should be recalled; they found that actively or passively immunised rabbits, which were protected against large doses of toxin given subcutaneously, contracted fatal tetanus after a small injection of tetanus toxin injected intracerebrally.

The Prophylactic Use of Tetanus Antitoxin.—The practice of giving antitoxin prophylactically in every case of a ragged, unhealthy-looking wound, especially when contaminated with soil, has been advocated. The principle has, for a considerable time, been applied in connection with the injuries contracted during the Independence Day celebrations in America, of which tetanus is a not uncommon sequel; a very definite fall in the death-rate has been thereby effected. It was during the late war, however, that the success of prophylaxis was established. During the early months, in the fighting on the Continent, tetanus was rife—its incidence in the wounded brought to Britain being about sixteen per thousand. After the autumn of 1914 prophylactic injections of antitoxin were given to every wounded man, with the result that the corresponding incidence was reduced to two per thousand. The initial dose was 500 U.S.A. units administered subcutaneously, but now 3000 I.U. are usually given. As passive immunity is of relatively short duration, the dose should be repeated at seven-day intervals till four doses have been given. Further, when at later periods operative interference, even with healed wounds, is necessary, a similar dose should be given, either subcutaneously forty-eight hours, or intramuscularly twelve hours, previous to the operation.

Attention has already been directed to the effects of the prophylactic use of antitoxin in modifying the clinical type of the disease.

Methods of Examination in a Case of Tetanus.—The occurrence of "drumstick" bacilli in stained smears of the discharge from a wound or in necrotic tissue excised from its margin is sugges-

tive, but other organisms with round terminal spores may occur (McIntosh). Again, the tetanus bacillus in the non-sporing condition is not characteristic in appearance. A much more certain method of diagnosis is the production of tetanus by a subcutaneous injection into mice of pathological material either directly or, preferably, as a filtrate from fluid anaerobic cultures. The latter, however, may yield negative results when there are other organisms present beside *B. tetani*. Accordingly, the organisms should be isolated in the pure state by culturing exudates or excised tissues from the wound by Fildes' method (p. 628). It must be remembered, however, that these procedures take too long a time to afford a useful guide for administration of antitoxin. The history of a contaminated wound should be the indication for treatment.

Bacillus Botulinus

One form of "meat poisoning" was shown by van Ermengem in 1896 to be caused by the toxin of an anaerobic bacillus to which he gave the name *Bacillus botulinus* (*Clostridium botulinum*). He cultivated the organism from a sample of ham, the ingestion of which in the raw condition had produced a number of cases, some of them fatal. It may be noted that the ham did not show signs of decomposition in the ordinary sense. The symptoms in these cases closely corresponded with those in the so-called "sausage-poisoning." They form a well-defined group, in which the symptoms are referable to the nervous system, and similar symptoms have been experimentally produced by means of cultures of the bacillus or its toxins. The chief symptoms of botulism are disordered secretion in the mouth and nose, more or less marked ophthalmoplegia externa and interna (dilated pupil, ptosis, etc.), dysphagia, and sometimes aphagia with aphonia, marked constipation and retention of urine, and in fatal cases interference with the cardiac and respiratory centres. Along with these there is practically no fever and no interference with the mental faculties. Vomiting may be an early symptom. The symptoms commence in the human subject usually twelve to twenty-four hours after ingestion of the poison. During recent years there have been many cases of botulism in America. The chief source has been home "canned" meats, fruits, and vegetables, which have been contaminated by the bacillus and in which it has grown and produced toxin. It also can flourish in meat pickled in brine if the NaCl content is under 8 per cent. Several small outbreaks have occurred in Great Britain, the source of the toxin being obscure in certain of those. *B. botulinus* has been cultivated from the intestines of animals, and it has been found to have a wide distribution in America in soils, both arable and

virgin. It has also been found in soil from various parts of the Continent and Great Britain.

Several different types of *B. botulinus* occur in nature. Two types designated A and B were first separated by the immunological differences of their toxins (*vide infra*); both cause botulism in man. A third type, C, was later defined, also D and E, but the distinction between them is not clear; these are associated with disease in various animals.

Microscopical and Cultural Characters.—*B. botulinus* is a bacillus of considerable size, measuring 3 to 8 μ in length and 0·5 to 0·8 μ in thickness; it has somewhat rounded ends and sometimes is seen in a spindle form. It is often arranged in pairs, sometimes in short threads. It is motile and has 4 to 8 lateral flagella of wavy form or they may be more numerous, according to the strain. It stains readily with the ordinary dyes, and is Gram-positive, though not strongly so. Spores are formed, which are oval in shape, terminal or sub-terminal in position, and only a little thicker than the bacilli. The spores may in certain circumstances withstand moist heat at 100° C. for one to six hours; in the moist state they withstand 107° C. for fifteen to eighty minutes, 115° C. for four to forty-two minutes, and 121° C. for two to twenty-two minutes (Dickson and others). The results of observations on the heat-resistance of spores have varied somewhat and apparently depend on several factors, *e.g.* the strain used, age of culture, hydrogen-ion concentration of the medium, etc.; further, only a small proportion of individual spores possesses the maximum resistance to heat, and results depend therefore on the number of spores in the test material.

The *B. botulinus* can be readily cultivated on the ordinary media, but only under strictly anaerobic conditions. A cooked-meat medium is suitable for cultivation (p. 59). Cultures in glucose agar resemble those of certain other anaerobes; there is abundant development of gas, and the medium is split up in various directions. In glucose gelatin a whitish line of growth forms with lateral offshoots, but liquefaction with abundant gas formation soon occurs. In gelatin plates the colonies after four to six days are somewhat characteristic; they appear to the naked eye as small semi-transparent spheres, and these on examination under a low power of the microscope have a yellowish-brown colour and are seen to be composed of granules which show a streaming movement, especially at the periphery. On agar, surface colonies are large and irregular with a thick central nucleus, a thinner periphery, and a fimbriate reticular border.

There is considerable variety in the colony appearances, however. The cultures have a rancid, though not foul, odour, due chiefly to the development of butyric acid. Coagulated serum or egg white is generally liquefied by types A and B, though not by type C. Similarly in cooked-meat medium types A and B digest the meat and darken it ; type C may produce no change. The fermentation of sugars and other carbohydrates varies with the type : A produces acid and gas from glucose, maltose, salicin, and glycerol ; B differs in the absence of salicin fermentation ; C does not ferment salicin or glycerol. The optimum temperature for strains studied by the earlier workers was from 20° to 30° C. ; but for other strains it is about 35° C.

Pathogenic Effects.—*B. botulinus* has little power of flourishing in the tissues, whereas it produces a very powerful filterable toxin when growing in food-stuffs. When a fairly large dose of toxin is injected subcutaneously in a guinea-pig, symptoms chiefly of paralytic nature may appear within about six hours and death follow within twenty-four hours. When the dose is sub-lethal a somewhat chronic condition may result in which local paralysis forms a striking feature. The characteristic effects can also be produced by oral administration of the filtered toxin, though in this case the dose requires to be larger. It is noteworthy that this toxin resists the gastric secretion and is absorbed by the stomach and small intestine without undergoing alteration—unlike the tetanus and diphtheria toxins. As in the case of the tetanus poison, the potency of the toxin is remarkable, the fatal dose by subcutaneous injection for a guinea-pig of 250 grams weight being in some instances 0·0001 c.c. of the filtered toxin or even less. When massive doses of toxin-free spores are given by the alimentary canal it has been found that multiplication of the organisms and toxin production may follow in certain cases, but there is no evidence that the disease in the human subject is produced in any other way than by the ingestion of previously formed toxin in the contaminated food. The presence of the toxin in infected ham was first shown by van Ermengem by inoculation with a watery extract, and a similar result has been repeatedly obtained by others in the case of other articles of diet which have produced the disease. The organism has been recovered from the organs of those who have died of the disease, but it is only after or immediately before death that a few bacilli may enter the tissues.

The properties of the botulinus toxin have been investigated, and have been found to correspond closely, as regards relative instability, conditions of precipitation, etc., with the toxins of

diphtheria and tetanus. It is inactivated by heating at 90° C. for twenty-five to forty minutes, or at 100° C. for four to twenty minutes. An antitoxin was prepared by Kempner by the usual methods, and was shown not only to have a neutralising property, but to have considerable therapeutic value when administered some hours after the toxin. These results have been fully confirmed. The subject was studied by Leuchs, and he found that the combination toxin-antitoxin can be split up by the action of acids and the two components recovered, just as Morgenroth showed to occur with diphtheria toxin (p. 221). The various types of the *B. botulinus* (*vide supra*) differ in the toxins which they produce. Thus the antitoxin to toxin A has no effect on toxin B, and *vice versa*. This is, of course, an important matter in relation to treatment, and a mixture of antitoxins for the A and B types has been introduced for therapeutic use in human cases. It has been found that in the eastern states of America most of the outbreaks have been due to type A, whilst in the western states type B has been more frequently found. A similar result has been obtained with regard to the distribution of the two types in the soil in these districts.

Botulism occurs in natural conditions in fowls and is known as "limber-neck"; in this affection the bacillus of type A has been found (Graham and Schwarze). Fowls are very susceptible to the toxin of type A, dying within twenty-four hours after oral administration, whereas they are more resistant to the toxin of type B. Type C may also be responsible (Bengtson). The latter type also causes a disease of ducks under natural conditions. Related to type C, although not identical with it, is type D which has been found to cause "lamsiekte," a disease of cattle in South Africa; and it has been reported under the name of "*B. parabotulinus*" as associated with a paralytic disease of cattle in Australia (Seddon). "Forage poisoning," an affection of equines in various parts of the world, has been observed in mules in South Africa and attributed to type E.

The condition of the nerve cells in experimental poisoning with the botulinus toxin was investigated independently by Marinesco and by Kempner and Pollack, and these observers agree as to the occurrence of marked degenerative changes, especially in the motor cells in the spinal cord and medulla. On the other hand, Dickson and others have found that the toxin has an important action on the vascular intima, leading to thrombosis in the small vessels; in this way the nerve cells may be damaged or necrosed. The work of Edmunds, Long and Keiper and others indicates, however, that the essential effect

of the toxin is on the nerve end-plates of muscles with paralyses of the vagus and parasympathetic nerve endings.

Laboratory Investigation of Botulism.—The investigation of cases and outbreaks of botulism is generally concerned with the examination of suspected articles of food. An extract in saline solution is made from the material and injected into mice or guinea-pigs. Control animals are also injected with a mixture of extract and a polyvalent antitoxin. Cultures are made from the food (previously heated at 60° C. for one hour to kill non-sporing organisms) in cooked-meat medium, and incubated for ten days at 35° C. The culture is filtered and the filtrate is injected into mice or guinea-pigs, control tests with antitoxin being also made as above. Dubovsky and Meyer have elaborated a special method with successful results for the cultivation and isolation of *B. botulinus* from food materials. The organism may also be recovered from the vomit or fæces of patients.

Anaerobes in Infected Wounds

It may be said that practically all such anaerobes come from the soil and that their original source is chiefly animal fæces. All cultivated soils are accordingly rich in such organisms. In the case of lacerated wounds contaminated by soil, and especially gunshot wounds, we have thus two main factors, the presence of damaged or necrosed tissues, and infection by various anaerobes of intestinal origin, though there are also some aerobes present from the same and other sources. At an early stage the number and variety of organisms, many of them spore-bearing, form a very striking feature. As reactive processes, exudation, leucocyte emigration, etc., come into play, we find in favourable cases that the anaerobes gradually diminish, while the aerobes continue to flourish, though in deep clefts and in necrosed tissue the former may persist for a long time. This change in the flora becomes more marked as suppuration becomes established and progresses : the ordinary pyogenic organisms multiply at the expense of the various bacilli, enterococcus, etc., till ultimately they are the chief organisms present. So far as serious complications are concerned, we may say that in the early stages these are chiefly due to the anaerobes, and in the later stages to streptococci and, to a less degree, to staphylococci. We have here to deal with the anaerobes, and these have the following effects : (*a*) poisoning by toxins, the outstanding example being of course the *B. tetani* ; (*b*) invasion of the tissues, the production of spreading œdema, necrosis, and gaseous emphysema—generally comprised under the term " gas gangrene " ; and (*c*) merely local inflammatory and putrefactive changes. The *B.*

tetani has already been dealt with, and it has been shown that it has no more infective or invasive properties than other saprophytic anaerobes. The number of anaerobes separated from wounds during the war was large, and with regard to them two general statements may be made. In the first place, only a few have been shown to cause by themselves definite spreading infections. Of these it is generally accepted that the *B. welchii* is by far the most important, next comes the "*Vibrion septique*," and then probably the *B. novyi* (*B. œdematiens*). In the second place, the organisms which sometimes cause these serious results may be present in wounds from which no complications arise. There must accordingly be favouring conditions in certain cases which lead to these grave and often fatal results. This, of course, holds with regard to bacterial infections in general, but it is especially well exemplified in the lesions in question. As possible determining influences, we might mention the degree of the injury, the dose, and possibly the virulence, of the invading organism, and the adjuvant effect of other organisms. And as shown by Bullock and Cramer (p. 174), the presence of soluble calcium salts has a very important effect in rupturing the defences and leading to infection by anaerobes. In the case of gas gangrene produced by the *B. welchii*, infection of lacerated muscle has been shown to be an extremely important factor in its origin and spread (*vide infra*).

This group of organisms (genus *Clostridium*) may be said to have the following characters. They are, on the whole, fairly large bacilli, easily stained and Gram-positive, though the occurrence of Gram-negative forms is fairly common in older cultures. Spore-formation is the rule; the spores, which are rounded or oval, have a thickness exceeding that of the bacillus, sometimes markedly so, and may be terminal, subterminal, or central in position. In a given species the position of the spore may vary somewhat, but in the case of some, *e.g.* the *B. tetani*, the spore is always terminal. The majority possess numerous peritrichous flagella, and many are actively motile; a few, *e.g.* the *B. welchii*, are non-motile. The earlier means of differentiation depended on morphological and cultural features in a few media, and on pathogenic effects. All these factors, however, vary somewhat. More recently the physical and chemical changes produced in various definite media have been added as a means of distinguishing them. The important work of M. Robertson, Henry, Wolf and Harris, and McIntosh may be mentioned in this connection. The appearances of superficial and deep colonies

have also been found of service. The result of biological inquiries has been to divide the organisms according to their metabolic activities into two main groups, namely, (1) those in which *saccharolytic* properties predominate, and (2) those in which *proteolytic* properties predominate. Variations are met with in the rapidity of the fermentation and also in the products which are ultimately formed. The recognition of these two main groups is of importance also from the pathological point of view, as the chief organisms which produce spreading lesions belong to the saccharolytic group. In fact there is often a "saccharolytic stage" of advancing infection, followed by a "proteolytic stage" of putrefaction. We shall give the chief characters of the most fully studied of these organisms, dealing first with the saccharolytic, which are the more important.

Bacillus welchii (B. aërogenes encapsulatus or Clostridium welchii)

This bacillus was first described by Welch and Nuttall in 1892, who showed that it was the cause of the extensive gaseous development which sometimes occurs in the organs *post mortem*, resulting in the formation of rounded gas cavities. It is now recognised that it is identical with an organism cultivated later by E. Fraenkel and called by him the *B. phlegmonis emphysematosæ*. The same bacillus was described by Veillon and Zuber, who gave it the name *B. perfringens*. During the war it came into great prominence, as it was proved to be by far the most important agent in the production of gas gangrene. We shall speak of it as *B. welchii*.

Microscopical Characters.—As seen in the serous fluid in a case of spreading gas gangrene, it is a comparatively large bacillus, measuring usually 4–6 μ in length (Figs. 142, 143) and relatively stout; but the thickness varies somewhat. Its ends are somewhat rounded, though those of some of the shorter forms are almost square. In cultures (Fig. 145) it is rather pleomorphous, and in sugar-free media there is a tendency to form filaments (Henry); again, short, almost coccus-like forms, may be met with. It is readily stained with the basic dyes, and is Gram-positive, though in older cultures Gram-negative forms occur. In the tissue fluids it usually has a distinct and fairly broad capsule—hence the original name (Figs. 144, 146); sometimes, however, no capsule is seen. In agar or broth media, again, no capsule is seen ordinarily, but in serum media it is

conspicuous. The organism is non-motile, and no flagella have been demonstrated.

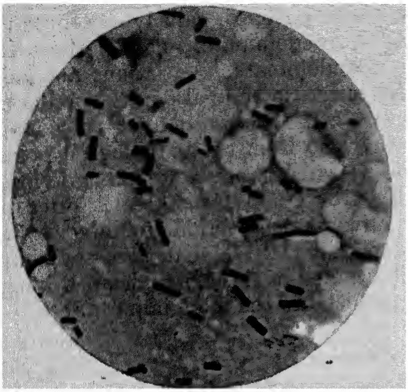


FIG. 142.—Film taken from margin of spreading gas gangrene, showing numerous examples of *B. welchii* (pure).[1]
Gram's stain. ×1000.

In the spreading area of the disease no spores are found though they have been described in the later stages when the bacillus is associated with other organisms. At first it was believed not to form spores, but spores are produced in serum media (Dunham); they are oval and fairly large, usually sub-terminal, occasionally central. In ordinary media, however, and in the presence of a trace of sugar, no spores are formed.

Cultural Characters.—*B. welchii* grows readily on various media, but only under fairly strict anaerobic conditions. It flourishes best at the temperature of the body, but grows also at room temperature. On serum agar the superficial colonies are usually circular in form, moist in appearance, with smooth margins, there being no radiate outgrowth or downgrowth (Fig. 147); the deep colonies are usually oval or lenticular in form, with sharp outline. It liquefies gelatin but not coagulated

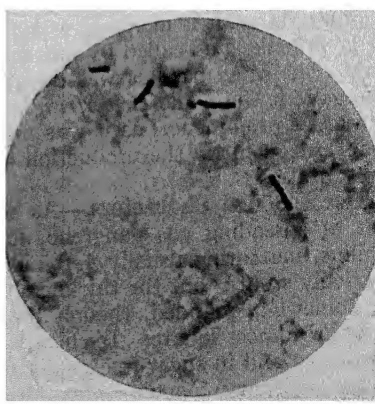


FIG. 143.—Film from necrosed muscle in gas gangrene, showing a few *B. welchii* with remains of muscle fibres.
Gram's stain. ×1000.

[1] We are indebted to Dr. J. W. McNee, for the preparations from which Figs. 142 and 143 were made.

serum. In milk the characters of the growth are of importance; it grows rapidly and leads to coagulation of the medium, the clot becomes broken up by gas bubbles—the so-called "stormy clot reaction"—and ultimately forms irregular tough masses bathed in comparatively clear whey. There is no digestion of the casein even after a long time. The culture has an odour of butyric acid. (Stormy fermentation of milk is also produced, however, by certain anaerobic butyric bacteria which form terminal oval spores—McCoy and others.) In cooked-meat medium *B. welchii* renders the meat a pink colour and produces a considerable amount of gas; there is a sour smell but no putrid odour or blackening of the medium. It produces acid and gas from glucose, maltose, lactose, saccharose, and starch, and sometimes from glycerol and inulin. Fermentation of these substances takes place with great rapidity, but the acid formed has a markedly deterrent action on the growth, and soon leads to its cessation. The *B. welchii* has thus very active saccharolytic action, whereas its digestive effect on proteins is slight, and these properties will be found to have an important bearing on its pathogenic effects.

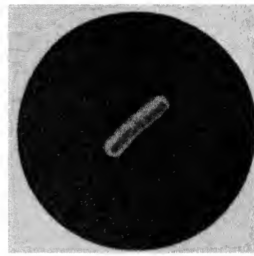


FIG. 144.—*B. welchii* showing capsule. Eosin capsule method. ×1000.

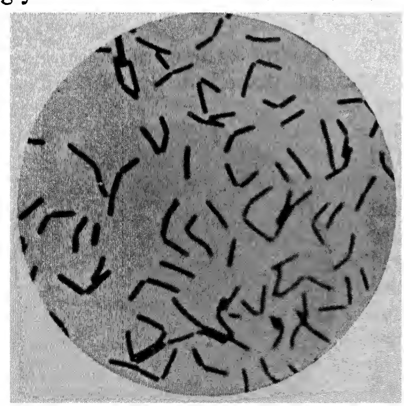


FIG. 145.—Film from a pure culture of *B. welchii*. Gram's stain. ×1000.

Pathogenic Effects.—In addition to invading the blood stream about the time of death, and giving rise to gas cavities in the organs, the *B. welchii* has been found in various emphysematous and gangrenous conditions in which the infection starts in connection with the alimentary canal. It is also, as has been indicated, by far the most important cause of gas

gangrene from war wounds. In this affection it is now recognised that the starting-point is usually some laceration of muscle, which has become contaminated with soil containing the bacillus. The spread of the disease is often remarkable, as cases have been recorded in which extensive emphysematous swelling with gangrene of a limb has occurred with a fatal result, well within twenty-four hours. In some cases the affection may be confined to individual muscles, and resection of these has been carried out, sometimes with success. Within a muscle, the necrotic change may affect individual fibres, leaving others in relation to them unaffected. The stages as described by McNee and Shaw Dunn are as follows. The bacilli spread with great rapidity along the interstitial tissue of the muscle, and may be found beyond the actual site of gangrene. They are present often in very large numbers and in practically pure culture. The fibres thus surrounded become somewhat swollen, altered in staining reaction, and separated from the interstitial tissue by a zone of serous fluid, poor in protein. The fibres then become completely necrosed, the sarcolemma nuclei losing their staining reaction, and about this time the fluid within the sarco-

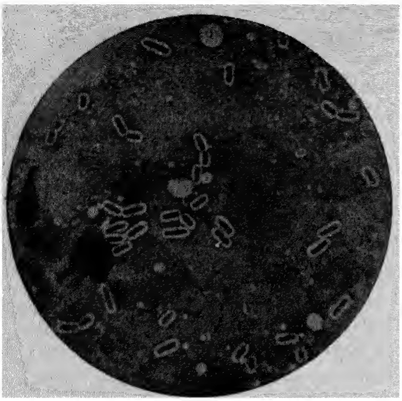


FIG. 146.—*Bacillus welchii*, showing capsules; film preparation from bone-marrow in a case where gas cavities were present in the organs. ×1000.

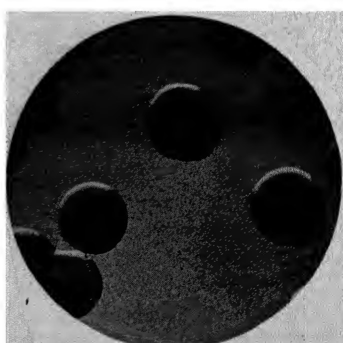


FIG. 147.—*B. welchii* surface colonies from anaerobic culture on glucose agar after forty-eight hours at 37° C. ×10.

lemma comes to contain the bacilli in large numbers. There is then evolution of gas, chiefly from the muscle carbohydrates, and the muscle substance becomes broken up and disintegrated, though the transverse striation may persist for a considerable time (Fig. 143). Finally the dead muscle may be invaded by other organisms, and become putrid and softened. Along with these changes in the muscle there occur œdema and emphysema in the interstitial and subcutaneous connective tissue, while the skin shows various kinds of discoloration, and the affected part is swollen, tense, and gives crackling on palpation.

To the naked eye the affected muscle is at first swollen and pale and has lost its elasticity; it soon assumes a brownish-red colour, is beset with gas bubbles and is putty-like in consistence, while later it becomes brownish-yellow, greenish, or dark red. It must be noted, however, that the bacillus does not cause the ordinary changes of putrefaction.

Wright found that locally there is a fall in the anti-tryptic action of the serum along with increased acidity, and he considered that the co-existence of these factors favours the growth of the bacilli and leads to their rapid spread. The spread of the bacilli is attended by practically no leucocyte reaction, unless when the invasion is becoming arrested or when it takes place around other organisms. The growth of the bacilli is essentially local, but they may enter the blood shortly before death, when they have been found in a certain proportion of cases. Instances have also been recorded in which they have settled in other parts of the body and produced lesions there—the so-called metastatic gas gangrene.

It has been observed that the number of *B. welchii* in the bowel may undergo great increase in certain conditions, *e.g.* the achlorhydria of pernicious anæmia (Davidson), intestinal obstruction, and it has been suggested that the toxin of the organism may be absorbed from the intestine and be responsible for pathological effects in these conditions. It is doubtful, however, whether *B. welchii* toxin is absorbed in an active state from the intestine.

Experimental Inoculation: Toxin and Antitoxin.—The virulence of the *B. welchii* varies considerably. In the case of many strains fatal gas gangrene may be produced in guinea-pigs by injection of cultures in fluid media, the bacterial invasion being favoured by the toxin present. The bacilli by themselves, for example when obtained from a surface growth on a solid medium, do not produce infection, but Bullock and Cramer showed that when an ionisable calcium salt is injected,

the bacilli quickly invade the tissues affected by the salt and cause a rapidly fatal infection. In the experimental disease the bacilli are abundant locally, but only a few are present in the blood stream. When the dose is sublethal a local gangrene may occur, with subsequent separation of the dead tissue; thereafter healing may rapidly follow. Intramuscular injection is the most effective method, especially in the rabbit, which is more resistant than the guinea-pig. The pigeon is found to be the most susceptible of the animals hitherto tested, the lethal dose being only a fraction of that for the guinea-pig. Injection into the pectoral muscle of a pigeon causes lesions in the muscle closely resembling those in gas gangrene in man, and death follows very rapidly, sometimes within a few hours. Bull and Pritchett succeeded in obtaining a true exotoxin. The medium used by them was plain meat broth containing fragments of sterile skeletal muscle of the pigeon or rabbit—autoclaved minced muscle may be used instead. After inoculation the medium is incubated under anaerobic conditions at 37° C. for twenty-four hours, and is then filtered through a Berkefeld N candle. The filtrate was found to be highly toxic for all the animals mentioned above, and gave rise to local lesions closely resembling those caused by the bacilli themselves. In addition to having a local necrotic effect on muscle, the toxin, or a moiety of it, is actively hæmolytic, and leads to a massive destruction of red corpuscles when injected intravenously. General toxic effects on muscle and on the nervous system are also observed. Accordingly, there is strong evidence that death from gas gangrene is due to a true toxæmia, and not to the production of acid in the tissues, as has been supposed by some. By means of injecting carefully graduated doses of the toxin, Bull and Pritchett produced an active immunity, and the serum of the treated animals possessed antitoxic properties. The antitoxin neutralises all the effects of the toxin in multiple proportions, and is protective and curative against infection with the bacillus in the pigeon. The antitoxin is titrated by its capacity to neutralise the toxin when tested by intravenous injection in mice (Hartley) or intramuscular injection in pigeons. The antitoxin has been used therapeutically in the human subject for the treatment of gas gangrene, gangrenous appendicitis and puerperal septicæmia; it has also been given prophylactically before operations on the alimentary or the genito-urinary tracts.

Variants of B. welchii have been isolated from an authentic strain which differed in form of colony, power of producing

toxin, microscopic and serological characters and sporulation (McGaughey).

Types of B. welchii pathogenic for sheep.—A number of diseases characterised by general toxic symptoms as well as local lesions in the intestines and elsewhere, have now been found to be caused by bacilli closely related to *B. welchii* Dalling showed originally that *lamb dysentery* is due to such an organism, *Cl. agni* (*Cl. welchii*, type B), which is actively toxigenic. There is evidence that *Cl. paludis* (*Cl. welchii*, type C) causes "struck," a disease of sheep in Kent in which necrosis of muscles is a feature (McEwen). "Infectious entero-toxæmia" of Australian sheep arises from absorption of toxins formed in the bowel by *Cl ovitoxicum* (*Cl. welchii*, type D), and this bacillus appears also to be responsible for "pulpy kidney disease" of lambs. In lamb dysentery, effective protection against the disease can be conferred by active immunisation of the pregnant ewes with toxin-antitoxin mixtures or formolised cultures of the causal organism, the antitoxin being transmitted to the lambs by the colostrum; alternatively the new-born lamb may be passively immunised with antitoxic serum. The chief differences between these organisms consist in the toxic products present in filtrates of fluid cultures, and each type of organism may form several distinct toxins. On the other hand, the same toxic constituent may be formed in common by two or more of the types. Wilsdon, by comparing the toxin-antitoxin relationships of various cultures, has differentiated three antigenic factors W, X, and Z. The antitoxin to type A fails to neutralise the toxins of types B, C, or D. On the other hand, antitoxins to types B, C, and D all are able to neutralise A-toxin. Accordingly, it may be concluded that A is the simplest type and its antigenic factor W is shared by the other types. Again, D toxin is neutralised by anti-B (as well as by anti-D), but not by anti-C; hence D contains a factor X which is also present in type B, but absent from type C. As the result of an extension of such tests to all the toxins and antitoxins along quantitative lines the distribution of the antigenic factors among the types has been ascertained and may be indicated as follows:

Type	Antigenic Factors
A	W exclusively.
B	Z chief, W distinct, X trace.
C	Z chief, W distinct.
D	X chief, W distinct.

Glenny and others have also distinguished a series of toxins. *Alpha* toxin is characteristic of the *Cl. welchii* (type A) responsible for gas gangrene in man; it possesses hæmolytic, necrotising and lethal properties. It is also formed by *Cl. agni* and *Cl. paludis*, as shown by the fact that it is neutralised by antisera to the two latter organisms. *Beta* toxin is necrotising and lethal but not hæmolytic; it is neutralised by antisera to *Cl. agni* and *Cl. paludis* but not by those to the other types. *Gamma* toxin, a constituent of *Cl. agni* cultures, is lethal to mice; it is not neutralised by alpha or beta antisera. Other toxins of these organisms have also been identified through their not being neutralised by antisera to the above toxins.

Bacillus fallax.—This organism was separated by Weinberg and Séguin, and the name was given by them on account of its resembling the *B. welchii*. It is smaller than the latter organism, being both somewhat thinner and shorter. It is Gram-positive, and in cultures forms spores which are usually subterminal in position. It possesses peritrichous flagella and is feebly motile. The growths resemble those of *B. welchii*, but the young surface colonies are more transparent and the older ones have a more irregular margin. The action on milk is much less marked, the formation of clot and gas usually occurring only after several days; the action on sugars also is feebler and more restricted. It has no digestive effect on gelatin, casein, or coagulated serum. It thus may be described as a non-proteolytic bacillus with somewhat weak saccharolytic action. Recent cultures produce a gelatinous œdema on injection into a guinea-pig; they, however, soon lose their virulence.

VIBRION SEPTIQUE (PASTEUR) (BACILLUS OF MALIGNANT ŒDEMA (KOCH) OR CLOSTRIDIUM ŒDEMATIS MALIGNI)

The *Vibrion septique* was first discovered by Pasteur in putrefying carcases. He described its characters and the lesions produced by it. He found that it grew only in anaerobic conditions, but pure cultures were not obtained. A similar organism was later more fully studied by Koch, who pointed out that the disease produced by it is not really a septicæmia, as immediately after death the blood may be free from the bacilli. Accordingly, he named it the "bacillus of malignant œdema." The two names are now used as synonymous, though it is not possible to say whether the organisms originally described by Pasteur and by Koch were identical. It is a common inhabitant of the intestine in animals.

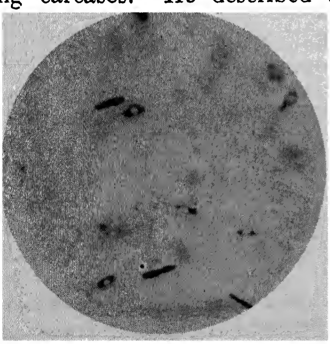


FIG. 148.—Film preparation from the affected tissues in a case of malignant œdema in the human subject, showing the spore-bearing bacilli. Gentian-violet. ×1000.

In pre-war times "malignant œdema" in the human subject was usually described as a spreading inflammatory œdema attended with emphysema, and ultimately followed by a certain amount of gangrene. In only some cases of this nature, however, is the bacillus of malignant œdema present, and it is usually associated with other organisms which aid its spread. In a fatal

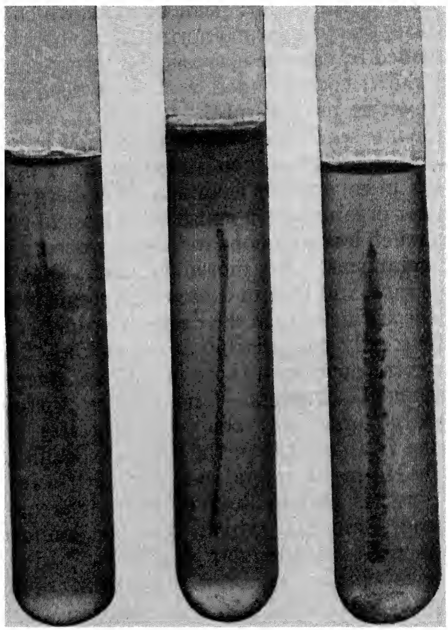
case, observed by Muir, in which the bacillus was present in pure condition, there occurred intense œdema with swelling and induration of the tissues, and the formation of vesicles on the skin. These changes were attended with a reddish discoloration, afterwards becoming livid. Emphysema was not recognisable until the very tense limb was incised, when it was detected, though in small degree. Further, the tissues had a peculiar heavy, but not putrid, odour. The bacillus, which was obtained in pure culture, was present in enormous numbers in the affected tissues, attended by cellular necrosis and serous exudation. The picture, in short, corresponded with that seen on inoculating a guinea-pig with a pure culture.

During the late war the organism was found in putrid wounds and cases of gas gangrene, being next in order of frequency to the *B. welchii* in gangrenous wounds. Weinberg places it along with the latter organism as a cause of "classical gas gangrene," though it is much less common, and usually occurs in association with other organisms. He, moreover, states that cases of pure infection are rare, and that in these emphysema is not a striking feature, gas occurring only in the deeper tissues in small bubbles, and sometimes only recognisable at operation. These features accordingly correspond with those in the case referred to.

Microscopical Characters.—The *Vibrion septique* is a comparatively large organism, being slightly less than 1 μ in thickness, that is, thinner than the anthrax bacillus. It usually occurs in the form of single rods 3 to 10 μ in length, but both in the tissues and in fluid cultures it frequently grows out into long filaments, which may be uniform throughout or segmented at irregular intervals. In cultures on solid media it chiefly occurs in the form of shorter rods with somewhat rounded ends. It can be readily stained by any of the basic aniline stains and is Gram-positive, but in older cultures Gram-negative forms occur. The rods are motile, possessing several peritrichous flagella. Motility is usually well marked in the serous exudate of the lesions, but in cultures only a few bacilli may show active movement. Under suitable conditions spores are formed which have an oval shape, their thickness somewhat exceeding that of the bacillus; they are central or subterminal in position. In acute spreading lesions the bacilli are usually free from spores, but at a later period they may be found, and large swollen lemon-shaped forms are often seen, the so-called "citron bodies." (Fig. 148).

Characters of Cultures.—This organism is a strict anaerobe;

it grows readily at room temperature, but the optimum is 37° C. In deep tubes of glucose agar at 37° C, growth is extremely rapid. Along the line of puncture, growth appears as a somewhat broad whitish line, with short lateral projections here and there (Fig. 149, B). Gas may be formed, but this is most marked in a shake-culture. The individual deep colonies are



A B C

FIG. 149.—Stab cultures in agar, five days' growth at 37° C. Natural size.

A Tetanus bacillus. B. Bacillus of malignant œdema. C. Clostridium chauvœi.

woolly in appearance without definite centre, whilst the superficial ones are thin discs with irregular peripheral radiations (Fig. 150). The growths generally are like those of the *B. tetani*, but have a somewhat coarser character. Cultures in gelatin present somewhat similar features, and the deep colonies have been compared to those of the *B. subtilis*; liquefaction of the medium follows. Coagulated serum is not liquefied. In cooked-meat medium the meat is reddened but there is no digestion.

The cultures possess a peculiar heavy, though not putrid, odour. McIntosh found that the organism ferments glucose, maltose, and lactose, but not saccharose, inulin, glycerol, or starch. His strains produced coagulation of milk, but without any digestion of the casein. The organism is thus mainly saccharolytic and only weakly proteolytic. Spore formation occurs in cultures above 20° C., and is usually well seen within forty-eight hours at 37° C.

Experimental Inoculation.—A considerable number of animals—the guinea-pig, rabbit, dog, sheep, and goat, for example—are susceptible to inoculation with this organism. There is general agreement as to its marked pathogenic properties. Especially is this the case when the serous exudate containing the bacillus is used for inoculation, a mere fraction of a cubic centimetre being a fatal dose. McIntosh found that with his strains 0·01 c.c. of a fluid culture injected intramuscularly killed a guinea-pig within twenty-four hours.

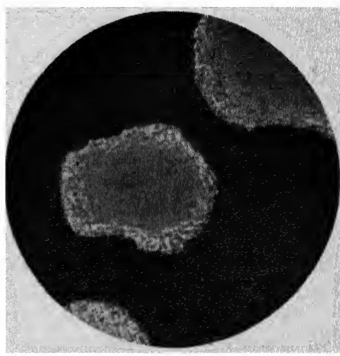


FIG. 150.—Vibrion septique, surface colonies from an anaerobic culture on glucose agar after forty-eight hours at 37° C. ×10.

Subcutaneous inoculation with pure cultures produces in the guinea-pig chiefly a widespread gelatinous œdema, and a blood-stained serous fluid exudes from the affected part. The underlying muscles are softened and partly necrosed, and of bright red colour; but there is little formation of gas, and putrid odour is almost absent. The internal organs show little change. The bacilli are present in the peritoneal fluid, and occur as long motile filaments. They have sometimes been cultivated from the blood, but they are always scanty. Infection with the organism is said to occur frequently when a little garden-earth is introduced subcutaneously in the guinea-pig, but in this case the local lesion presents a putrid character, owing to the presence of other organisms.

When the bacilli are injected into mice, however, they enter and multiply in the blood stream, and they are found in considerable numbers in the various organs, so that a condition not unlike that of anthrax is found. The spleen also is much swollen.

Toxin.—An exotoxin can be obtained in fluid cultures under suitable conditions. Of a potent toxin 0·1 c.c. injected intravenously into a rabbit will cause death in three to fifteen minutes, with respiratory disturbance, paralysis, and convulsions. Guinea-pigs and mice are also killed by the toxin given intravenously. Intramuscular or subcutaneous injections cause local œdema, red staining of the tissues, and some necrosis. Intracutaneous injection of the toxin in guinea-pigs causes a necrotic lesion. *In vitro* the toxin causes contraction of smooth muscle; it also has hæmolytic action.

Immunity.—Malignant œdema was one of the first diseases against which immunity was produced by injections of toxins. The filtered cultures of the bacillus in sufficient doses produce death with the same symptoms as those caused by the living organisms, but a relatively large quantity is necessary. Roux and Chamberland (1887) found that if guinea-pigs were injected repeatedly with non-fatal doses of cultures sterilised by heat or freed from the bacilli by filtration, immunity against the living organism could be developed in a comparatively short time. They found that the filtered serous exudate of animals dead of the disease is more highly toxic, and also gives immunity when injected in small doses. The serum of immunised animals contains antitoxin, which can be titrated according to its capacity to neutralise the action of the toxin as tested by intravenous injection in mice or intracutaneous injection in guinea-pigs (Hartley and Bruce White). The antitoxic serum protects effectively against inoculation with the living bacilli or washed spores *plus* calcium chloride; it also has curative action (Craddock and Parish). The antitoxin has been used therapeutically and prophylactically in the human subject.

Braxy, a disease of sheep, has been found to be due to infection by *Vibrion septique* (Gaiger). In this condition there is an intense inflammatory lesion with œdema, hæmorrhage, and necrosis, starting in the wall of the fourth stomach and tending to spread to neighbouring parts of the alimentary tract. The bacilli can be demonstrated in the affected tissues. Little is known, however, regarding the factors that predispose to this infection. Culture-filtrates, toxin-antitoxin preparations, and formolised whole cultures ("anaculture") have been utilised with success in the prophylactic immunisation of sheep.

Bacillus chauvœi (*Clostridium chauvœi*).—This bacillus, so far as is known, never infects the human subject. It causes quarter-evil (German Rauschbrand; French, Charbon Symptomatique). The natural disease, which occurs especially in certain localities, affects chiefly young cattle, also sheep. Infection takes place by some wound of the surface, and then spreads in the region around, attended by inflammatory swelling, bloody œdema, and emphysema of the tissues. The part becomes greatly swollen, and of a dark, almost black, colour. Hence the name "black-quarter" by which

the disease is often known. The bacillus is present in large numbers in the affected tissues, associated with other organisms, and also occurs in small numbers in the blood of internal organs. Morphologically it closely resembles *V. septique*. It is, however, generally somewhat thicker, and does not usually appear in long filaments when films from the surface of the liver of an infected guinea-pig are examined; occasionally it occurs in short chains. The characters of the cultures, also, resemble those of the bacillus of malignant œdema. Stress was at one time laid upon certain differences in cultural characters for distinguishing this organism from *V. septique*. Weinberg has concluded, however, that it bears to *V. septique* a similar relation to that which the types of *B. welchii* pathogenic for sheep do to the human type.

The disease can be readily produced in various animals, *e.g.* guinea-pigs, by inoculation with the affected tissues of diseased animals, and also by means of pure cultures, though an intramuscular injection of a considerable amount of the latter is sometimes necessary. The condition produced in this way closely resembles that in malignant œdema. The disease is one against which immunity can be developed in various ways, and methods of preventive inoculation have been adopted in the case of animals liable to suffer from it, *e.g.* by injection intravenously or intraperitoneally of a non-fatal dose of the œdematous fluid ("aggressin") from the tissues of infected animals which contain the bacilli, or by injection of larger quantities of this material attenuated by heat, drying, etc. (Arloing, Cornevin and Thomas, and others). Cultures attenuated by heating or products of the bacilli obtained by filtration of cultures have also been used. An antitoxin has been produced against the toxins of the bacillus, and this combined with injection of the organisms has also been employed. The antitoxin is said to increase the chemotactic properties of the leucocytes.

Bacillus novyi (*Clostridium œdematiens*).—This organism, first described by Weinberg and Séguin, has the following characters. It is a fairly large bacillus, of about the same size as the *B. welchii*, but tending to be rather longer. It is distinctly pleomorphous, often occurring in C and S forms, and growing also in chains. It is Gram-positive, but Gram-negative forms are found in older cultures. It possesses numerous lateral flagella, though in ordinary conditions motility is practically absent. Spore formation occurs, the spores being usually subterminal in position.

Cultivation.—The organism grows well on all the ordinary media down to a temperature of about 20° C., but only under strict anaerobic conditions. On solid media the deep colonies are small, somewhat irregular balls with woolly margin, while the superficial ones are film-like with wavy border. In milk it causes the formation of a loose acid clot, which falls to the bottom as a grumous deposit; there is no digestion of the casein, nor is there any digestion of coagulated serum. Gelatin, however, is liquefied. It actively ferments nearly all the ordinarily used sugars with evolution of gas; but according to other observers only glucose, lævulose, and maltose are attacked. In cooked-meat medium it produces a pink colour, which soon fades, and there is slight formation of gas. The bacillus may thus be regarded as belonging to the saccharolytic type of anaerobes.

Pathogenic Effects.—In a series of cases of gas gangrene Weinberg found the *B. novyi* to occur next in order of frequency to the *B welchii*, and he considers it to be the most important agent in what he calls the "toxic form" of gas gangrene. This type is characterised by a rapidly spreading gelatinous œdema, with little or no gas formation, and by severe symptoms of general poisoning. Blood culture usually gives a negative result, though the bacillus may be found in the blood after death. Intramuscular or subcutaneous injection in the guinea-pig gives a similar picture, the chief feature being the extent and thickness of the œdema; the lesion has no putrid odour. In sheep in Australia this organism causes "black disease," an infectious necrotic hepatitis (Albiston) The organism was shown by Weinberg to form a soluble toxin, which in the case of some strains is very potent. Injection of a filtrate from a fluid culture reproduced the characteristic œdema in the guinea-pig. He also produced an antitoxin which was efficient when tested experimentally, and which was used in some cases of the human infection, apparently with success. The antitoxin is titrated by its capacity to neutralise the lethal action of the toxin as tested by intramuscular injection in mice (Walbum and Reymann).

Bacillus tertius (*Clostridium tertium*).—This is another saccharolytic bacillus, but with *terminal spores*. It is common in contaminated wounds, and the name was given by Henry, as he found it to be third in order of frequency among the anaerobes. It is regarded as being probably the same as the bacillus IX of von Hibler and the bacillus Y of Fleming. The *B. tertius* is a fairly long and thin bacillus, and is often somewhat curved; it is Gram-positive, but the power of retaining the stain is soon lost in cultures. It is feebly motile or non-motile. The spores are terminal; the small forms are round, and stain deeply with a basic dye; the larger are oval, racquet-shaped, sometimes of considerable length. Occasionally a spore is present at each end of a bacillus. The superficial colonies are round, semi-transparent discs, which do not become large; the deep colonies are of lenticular shape; occasionally, from both, small offshoots occur. On a moist surface there is a tendency for the growth to spread as a thin film. In milk a small amount of gas is produced, and a day or two later a soft friable coagulum. In cooked meat medium both acid and gas are formed; later the fluid becomes clear and the meat assumes a pink colour. There is no liquefaction of gelatin or coagulated serum. The organism has wide fermentative action when tested on various carbohydrates, but different strains vary in this respect. It has practically no pathogenic effects when tested experimentally, though it probably gives rise to gas-formation in wounds.

Bacillus sporogenes and *B. histolyticus* are typical examples of sporing anaerobes in which proteolytic properties predominate.

Bacillus sporogenes (*Clostridium sporogenes*).—This organism, which was first separated from fæces by Metchnikoff and described by him, is probably the commonest anaerobe in cultivated soil. It is present in the great majority of putrid wounds, and, owing to its rapid growth and spore formation, often interferes with the

separation of other anaerobes. It is a fairly large bacillus, of about the same length as the *B welchii*, but thinner, and usually occurs as single elements. It is Gram-positive, but, as is common with members of the group, Gram-negative forms are to be found in older cultures. Spore-bearing forms are common in wounds, and in cultures spores are formed with great rapidity, so that they may be seen within twenty-four hours (Fig. 151). The spores, which have very high powers of resistance, are usually subterminal, though occasionally central in position. The organism possesses numerous peritrichous flagella, and most strains are actively motile. It grows readily under anaerobic conditions, and the cultures have a markedly putrid odour. In deep glucose agar tubes the growth forms a thick line, from which there are short and stout lateral offshoots, attended by abundant gas formation, while individual colonies are small balls with woolly margin. Superficial colonies have a granular centre and present an arborescent appearance at the edge. The organism rapidly liquefies gelatin and coagulated serum, and also pieces of coagulated white of egg. In cooked-meat medium there is evolution of gas and rapid digestion; the meat assumes a dirty, purplish tint, and ultimately becomes blackened. In milk there is a precipitation of casein without actual coagulation, and then digestion rapidly follows. The organism ferments glucose, lævulose, and maltose, but none of the other sugars ordinarily used. The organism is thus seen to have marked proteolytic properties, and it has been shown to form amino-acids, and as final products ammonia, sulphuretted hydrogen, and various volatile substances. It forms large quantities of butyric acid even in sugar-free media (Wolf and Harris).

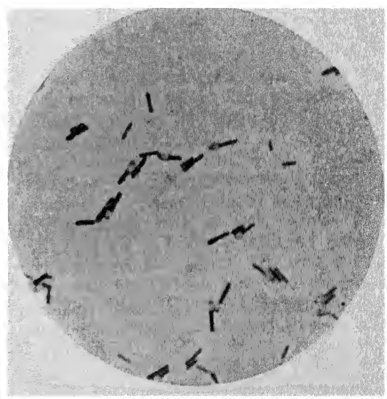


FIG. 151.—*B. sporogenes*, pure culture, showing subterminal spores. Stained with carbol-thionin. ×1000.

The *B. sporogenes* has little or no pathogenic properties when injected in animals; and observations on gun-shot wounds supply no evidence that it invades the healthy tissues. It may be regarded chiefly as a proteolytic saprophyte which grows on dead and dying tissues and brings about digestive softening and putrefactive changes. The *B. sporogenes* is closely allied to another proteolytic and putrefactive anaerobe described by Bienstock under the name *B. putrificus*.

Bacillus histolyticus (*Clostridium histolyticum*).—This is another proteolytic and putrefactive anaerobe separated by Weinberg

from cases of gas gangrene. It is 2–6 μ in length and rather thinner than the *B. welchii*; it is often arranged in pairs. It is Gram-positive and forms large oval subterminal spores. The surface growth is in the form of a very thin film, with offshoots at the margin. Its action on milk and coagulated serum is similar to that of the *B. sporogenes*, but is even more rapid. In cooked-meat medium also it produces very rapid digestion without odour, and one feature described by Henry is the separation of white balls of acicular crystals which are probably tyrosine—an appearance which is apparently characteristic of this organism. A striking evidence of the proteolytic action of this organism is seen when it is injected subcutaneously in a guinea-pig. A rapid digestion of the tissues in the vicinity occurs, so as sometimes actually to expose the bones. A soluble toxin is formed.

Other organisms of the group of sporing anaerobic bacilli which have been described in gas-gangrene are *B. parasporogenes*, *B. bifermentans*, *B. aerofetidus*, and *B. multifermentans tenalbus*.

B. parasporogenes differs from *B. sporogenes* in producing a smooth round surface-colony, and can also be distinguished by agglutination reactions with antisera (McIntosh and Fildes).

B. bifermentans is mainly proteolytic. It is non-motile and resembles *B. welchii* in morphology. Spores are formed readily and are oval and subterminal. Surface colonies are round or crenated; deep colonies are without woolly outgrowths. In other characters it resembles *B. sporogenes*.

B. aerofetidus is also essentially proteolytic. It appears as a slender bacillus 2 to 4 μ in length, which is only slightly motile and tends to stain Gram-negatively. Spores are not readily produced. When seen they are subterminal in position. Surface colonies are small (1 to 2 mm. in diameter), round, and transparent.

B. multifermentans tenalbus possesses saccharolytic but no proteolytic properties (like *B. fallax*). In morphology it resembles *V. septique* but differs in its lack of pathogenic action when injected into animals.

The following organisms belonging to the group have also been described:

B. cochlearius is a motile bacillus showing oval terminal spores, and differs from other members of the group in possessing neither saccharolytic nor proteolytic properties.

B. tetanomorphus is of interest in its close morphological similarity to *B. tetani*. Surface colonies are small, more or less round, and transparent. It is non-pathogenic to laboratory animals. It possesses saccharolytic but no proteolytic properties.

B. sphenoides also tends to resemble *B. tetani* in morphology when the spores are fully developed. The bacilli are motile and may appear fusiform in shape. Surface colonies are round and without projections. It possesses saccharolytic but no proteolytic properties.

Fusiform Anaerobic Bacilli

Bacillus fusiformis (*Fusiformis fusiformis*).—Babés in 1884 described organisms of this type in a diphtheria-like

affection of the fauces, and since that time the presence of similar organisms has been noted in necrotic inflammations, ulcerative stomatitis, noma, and like affections. They have also been found in pulmonary lesions and in abscesses in other parts of the body; in these the pus is very foul-smelling. The association of fusiform bacilli with a form of angina has been specially recognised since the work of Plaut and of Vincent; and this condition often goes now under the name of "Vincent's angina." He recognised two forms of the affection—(*a*) a diphtheroid type, characterised by the formation of a firm yellowish-white false membrane, very like that of diphtheria, associated with only superficial ulceration; and (*b*) an ulcerative type, where the membrane is soft, greyish, and foul-smelling, attended with ulceration and surrounding œdema. In the former type fusiform bacilli may be present alone; in the latter, which is distinctly the commoner, there are also spirochætes. The fusiform bacilli are thin rods measuring on the average 10 to 12 μ in length, and less than 1 μ in thickness; they are straight or slightly curved, and are tapered at their extremities. The central portion often stains less deeply than the extremities, and not infrequently shows unstained points and granules (Fig. 152; Plate I., Fig. 4). The organisms are non-motile. They stain fairly deeply with Löffler's methylene-blue or with weak carbol-fuchsin, and are Gram-negative. The spirochætes are long delicate organisms showing several irregular curves, and are motile; in appearance they resemble the *Treponema refringens* and similar organisms found in gangrenous conditions. They stain less deeply than the bacilli. Sometimes they are numerous, sometimes scanty. In a section through the false membrane, when stained with methylene-blue or thionin, there is usually to be seen a darkly stained band, a short distance below the surface, which is due to the presence of large masses of the fusiform bacilli closely packed together; neither they nor the spirochætes appear to pass deeply into the tissues. It is also to be noted that fusiform bacilli are sometimes present in the secretions of the mouth in normal conditions, and may occur in increased numbers in true diphtheria. Vincent's results have been confirmed by others, and there is no doubt that fusiform bacilli, of which there are probably several species, are associated with various spreading necrotic lesions. During the war, cases of Vincent's angina were of common occurrence and were met with in small epidemics. Ulcerative gingivitis and stomatitis have been found to be associated with the presence of the same organisms, and in some cases these lesions precede the

infection of the fauces. It would be advisable to apply the term "Vincent's disease," as suggested by Bowman, so as to include all the lesions produced by the organisms in question. In phagedænic lesions of the genitalia, fusiform bacilli are usually present, with or without spirochætes, though in our experience they are as a rule of smaller size than those met with in the throat. Cultures of fusiform bacilli have been obtained by Ellermann; Weaver and Tunnicliff; Smith; and others. They grow only under anaerobic conditions, and the best media are

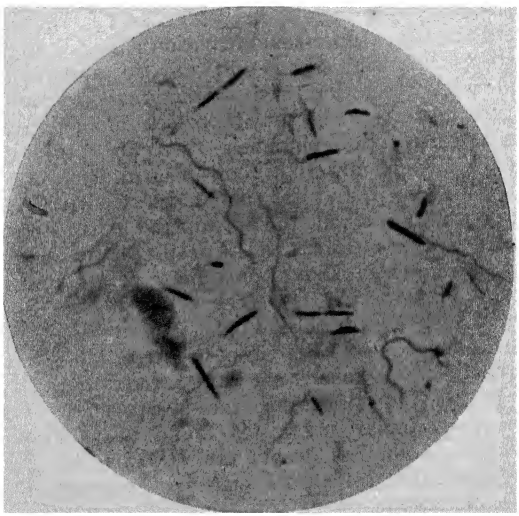


FIG. 152.—Film preparation from a case of Vincent's angina, showing fusiform bacilli and spirochætes. Stained with weak carbol-fuchsin. ×1000.

those consisting of a mixture of serum or blood and agar (1 : 3). The organisms form small rounded colonies of whitish or yellowish colour, somewhat like those of a streptococcus, but rather felted in appearance on the surface. In cultures, especially in fluid media, undulating filamentous forms may be found, but there does not appear to be sufficient evidence that the fusiform bacilli and the spirochætes found in angina are stages in the development of the same organism, as was maintained by Tunnicliff. Krumwiede and Pratt, for instance, found that the filamentous forms were not true spirochætes but merely

represented variations in morphology. These observers studied the fermentative properties of the *B. fusiformis* and found that it fermented various sugars without the formation of gas ; they distinguished two types, one fermenting saccharose, the other not. Knorr has distinguished three types. Injections of pure cultures in animals sometimes produce suppuration but never necrosis (Ellermann). It is probable that there exist a group of these organisms ; but difficulties in their isolation and cultivation have hindered exact study of them.

CHAPTER XXI

PATHOGENIC SPIROCHÆTES: TREPONEMA PALLIDUM AND TREPONEMA PERTENUE

Introductory.—The general morphology of the spirochætes and their resemblance in certain particulars to protozoa have already been discussed (pp.2, 31). In some instances pathogenic species are transmitted directly, but in others blood-sucking ectoparasites are concerned and it has been suggested that some phase of a supposed life cycle may occur in the intermediate arthropod host. The parasitic members of the order may be classified into the two main genera, *Treponema* and *Leptospira* (Dobell). The former, which show undulating motion, vary in their curving in different species; some present the appearance of a perfect and regular spiral, *e.g.* the *Treponema pallidum* of syphilis. To this type the term "Treponema" has been restricted by the Society of American Bacteriologists. In others, *e.g.* the organisms of the relapsing fevers, the coils or spirals are less numerous and of greater amplitude and more open; in stained films, instead of showing a definite spirality the organisms appear as filaments with curves or undulations. Organisms of this second type have been placed in a separate genus, *Borrelia.* The organisms of the genus *Leptospira* exhibit a very delicate spiral structure with numerous fine coils which are best seen by dark-ground illumination and may not be so apparent in stained preparations. The leptospira possesses characteristic hooked ends, which when in rotatory movement appear as bulbous swellings or eyelets, while the rest of the organism tends to remain rigid.

The human diseases produced by spirochætes—the *Spirochætoses*—can be classified into certain main groups: (1) syphilis and yaws, (2) the spirochætal relapsing fevers, (3) certain ulcerative and gangrenous conditions with which spirochætes are associated, usually along with a characteristic fusiform bacillus (*B. fusiformis*), *e.g.* Vincent's angina, gangrenous balanitis, etc., (4) infectious jaundice (Weil's disease), due to a leptospira, (5) rat-bite fever, due to a spiral organism which appears

to be more related to the spirilla, though it has usually been classified with the spirochætes. Certain of these diseases are essentially blood infections, *e.g.* the relapsing fevers, and the spirochæte is transmitted by blood-sucking arthropods. Others are due to organisms which are primarily tissue parasites, *e.g.* the *Treponema pallidum*—blood invasion, when it occurs, being a secondary phenomenon—and infection occurs by direct contact. Infectious jaundice seems to occupy a somewhat intermediate position, as the organisms occur in the blood stream, but tend to settle and flourish in certain organs.

It should also be noted that certain types of spirochætes are common commensal organisms and some of them resemble pathogenic types. These will be referred to in relation to the pathogenic types which they resemble.

Treponema Pallidum

The cause of syphilis is the organism discovered by Schaudinn and Hoffmann in 1905 and called by them the *Spirochæta pallida*, now known as the *Treponema pallidum*. They described its characters and its occurrence in syphilitic lesions, and their observations have been fully confirmed. Recognition of this organism was long delayed owing to the difficulty in distinguishing it in the fresh state on examination by transmitted light. Further, it has a weak and variable affinity for stains ; for its demonstration prolonged treatment with one of the Romanowsky solutions oı staining after the application of a mordant is required (p. 129). Then the apparent thickness of the spirochæte depends on the intensity of staining and this may introduce a difficulty in distinguishing it from other similar organisms. Accordingly, the best method of detecting it is by dark-ground examination (p. 107). In order to demonstrate *Tr. pallidum* in sections advantage is taken of its affinity for compounds of silver (p. 126).

Microscopic Characters.—*Tr. pallidum* (Schaudinn and Hoffmann) is a minute organism of spiral shape, showing usually from six to fourteen curves, though longer forms are met with ; the curves are small (each measuring a little over 1 μ in depth and breadth), comparatively sharp, and regular (Figs. 153, 154). It measures 4 to 14 μ in length, while it is extremely thin, its thickness being only 0·25 μ. In a fresh specimen, say, in exudate from a chancre, the organism shows active movements, which are of three kinds—rotation about the long axis, gliding movements to and fro, and movements of flexion of the whole

body; there is little actual locomotion, and a spirochæte will often remain in the same field for a long time, although the organisms as seen in the testicular lesion of experimentally infected rabbits may show active movements of progression. Each end consists of a straight terminal filament considered not to be a flagellum. In fresh specimens and in dried films (Figs. 153, 154) the regularity of the spirals is well maintained, though in the latter there is sometimes distortion or drawing out of a spiral. When stained by any of the Romanowsky solutions, *e.g.* Giemsa's or Leishman's, it appears of a faint pink tint; also it is thinner than when stained by Becker's method.

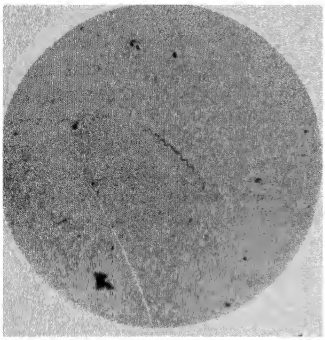


FIG. 153.—Preparation from exudate of hard chancre showing *Treponema pallidum*. Giemsa's stain. ×1000.

In the microscopical diagnosis of the organism of syphilis, just as in the case of the tubercle bacillus, an all-important point is the source of the organism; and we may say that, excepting the case of yaws, which does not occur in this country, an organism with the characters described above can be identified with certainty as the *Tr. pallidum* provided that it is obtained from the *substance* of the tissue lesion.

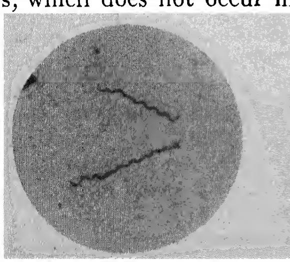


FIG. 154.—Film preparation from exudate of hard chancre showing *Treponema pallidum*. Becker's stain. ×1500.

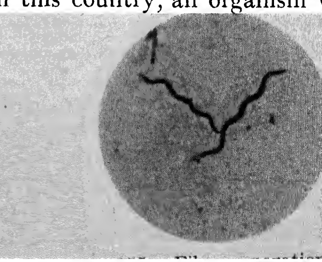


FIG. 155.—Film preparation from balanitis showing *Treponema refringens*. Becker's stain. ×1500.

Strains of Tr. pallidum with varying morphology.—Noguchi, on studying different strains of the *Tr. pallidum* in cultures, found

that they varied in thickness, and he was able to distinguish thick, thin, and intermediate types. He also found that they differed in their pathogenic action, the thick forms on injection into the testicle of a rabbit causing nodular lesions of cartilaginous hardness, the thin forms producing a diffuse indurative lesion. Levaditi and Marie also hold that there are "dermotropic" and "neurotropic" strains of *Tr. pallidum*, which differ in their pathogenic properties. These observations are suggestive as possibly throwing some light on the variations in the effects in the human subject; but they have not been confirmed by others.

Levaditi and others have postulated a cycle of development of *Tr. pallidum* in order to explain the high infectivity of tissues in which no spirochætes can be detected on microscopical examination, *e.g.* the lymph glands of inoculated rabbits after the infection has become latent. They conclude also that a similar cycle occurs on experimental inoculation of the central nervous system with *Tr. pallidum*. One stage in the cycle is believed to be ultramicroscopic. While those views would explain certain difficulties in connection with the pathology of neurosyphilis, there is no definite evidence supporting them

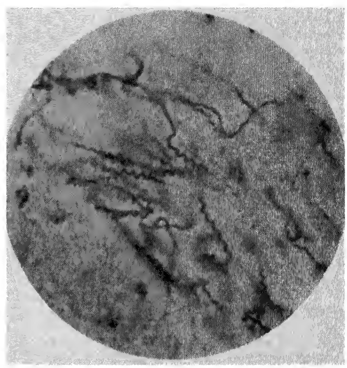


FIG. 156. — **Section of** liver from a case of congenital syphilis, showing numerous examples of *Treponema pallidum*. Levaditi's method. ×1000.

Other spirochætes which occur on mucous surfaces and in ulcerated lesions. — In ulcerated syphilitic lesions, and also in non-syphilitic lesions of the genitalia, other organisms are, of course, present, and not infrequently various other spirochætes; different varieties of the latter may also be met with normally on certain parts of the body surface, *e.g.* in the mouth and on the genitalia in both sexes, also in the fæces. Of these, several species have been described, e.g. *Tr. refringens*, *Tr. gracile*, *Tr. minutum*. *Tr. refringens* is a comparatively coarse organism, more highly refractile, while its curves vary during the movements; in film preparations the curves appear irregular or are lost to a large extent (Fig. 155). It is similar in appearance to the spirochætes of Vincent's angina (p. 663). It is found on ulcers on various parts of the body, and may occur in the sputum in cases of bronchitis. Some of the other species resemble *Tr. pallidum* more closely in the regularity of their curves, e.g. *Tr. gracile* (Fig. 158), but are larger or differ in the character of

their movements. *Tr. minutum,* isolated by Noguchi from the secretions of the external genitalia, resembles *Tr. pallidum* closely. We believe that in the case of genital lesions there is little difficulty to the experienced observer in recognising the *Tr. pallidum,* provided that the superficial organisms are removed and the lymph is taken from the lesion for examination. Also, these organisms generally stain deeply with Giemsa's stain and are of a bluish tint, thus contrasting with the faint pink of *Tr. pallidum.* In lesions of the mouth and probably in some others, *e.g.* fœtid ulcerations, etc., and also normally in the mouth (between the teeth) there occur, however, spirochætes which are indistinguishable morphologically from the *Tr. pallidum, e.g.* the *Tr. microdentium* (Fig. 157) and *Tr. mucosum.* These may be specially abundant in carious teeth and pyorrhœa alveolaris. Both of these organisms have been cultivated by Noguchi; they have been proved to be devoid of pathogenic properties, and the cultures, moreover, have a foul odour.

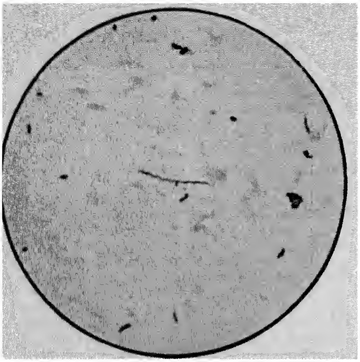


FIG. 157.—*Tr. microdentium.* Film preparation from mouth showing characteristic morphology of the organism. Stained by Becker's method. ×1000.

FIG. 158.—*Tr. gracile* (*Tr. calligyrum*). Film preparation from balanitis pus treated by India ink method. The organism, with regular spirals, is seen in the centre of the field. ×1500.

Distribution of Tr. pallidum in the tissues.—As regards the distribution of *Tr. pallidum,* the following is a summary of the results of many investigations. In the primary sore in the

stage of active ulceration and in the related lymphatic glands, the pulp of which can be conveniently obtained by means of a hypodermic syringe, the organism has been found in a very large majority of cases. It has been also obtained in the papular and roseolar eruptions, in condylomata and mucous patches—in fact, one may say generally, in all the primary and secondary lesions. Schaudinn in his last series of cases, numbering over seventy, found it in all, and on a few occasions detected it in the blood during life in secondary syphilis. It has also been obtained from the spleen during life. In the congenital form of the disease the organism may be present in large numbers (Plate II., Fig. 6), as was first shown by Buschke and Fischer, and by Levaditi. In the pemphigoid bullæ, in the blood, in the internal organs—the liver, suprarenals, lungs, spleen, and even in the heart—its detection may be comparatively easy, owing to the large numbers present (Fig. 156). In the organs in congenital syphilis the spirochætes are chiefly extra-vascular in position, but many may occur in the interior of the more highly specialised cells, for example, liver cells. They also abound sometimes on mucous surfaces, *e.g.* of the bladder and intestine in cases of congenital syphilis. The enormous numbers of the organism which may be present in a well-preserved condition in macerated fœtuses render it probable that the organism may multiply in the dead tissues under anaerobic conditions. It is also present in syphilitic placentæ, though not usually in large numbers. It has been generally supposed that tertiary syphilitic lesions are non-infective, and the results of the earlier observations on the *Tr. pallidum* were apparently in accordance with this view, as they gave negative results. More prolonged search has, however, shown that the organism may occur in tertiary lesions also. It has been found to be present in the peripheral parts of gummata, especially at an early stage of their formation ; and the observations of Schmorl, Benda, J. H. Wright, and others show that it is often to be found in syphilitic disease of arteries, sometimes occurring in considerable numbers in the thickened patches in the aorta. That the spirochæte may persist in the body for a very long time after infection, has been abundantly shown by different observers ; in one case, for example, its presence was demonstrated sixteen years after the primary lesion. Noguchi and Moore discovered the spirochæte in the brain in general paralysis of the insane in a certain proportion of cases. The organism was seen in all the layers of the cerebral cortex, with the exception of the outermost, and the cases in which it was

found had run a relatively rapid course. It occurs in scattered clumps which may be very hard to find (Fig. 159). Infection has also been transmitted to the rabbit (*vide infra*) by inoculation with the brain tissue of general paralytics ; but as a rule only negative results have been obtained in such experiments.

Cultivation.—Although Mühlens and Hoffmann had previously obtained pure cultures of an organism morphologically identical with the *Treponema pallidum*, it is chiefly to Noguchi that we owe the methods of cultivation. We shall accordingly state his results, which in certain respects differ from those of the other two observers. In the first instance his cultures were made from syphilitic lesions in the rabbit, but later directly from the lesions of the human disease. As a culture medium he used a mixture of 2 parts of 2 per cent. agar and 1 part of ascitic or hydrocele fluid, to which a small portion of sterile rabbit's kidney or other organ was added, the medium being placed in deep tubes and covered with a thick layer of paraffin oil or vaseline. The medium was inoculated through the oil, the maintenance of strict anaerobiosis being essential. The contaminating bacteria which were present formed a thick growth along the line of inoculation, whilst the spirochætes grew as a diffuse haze into the surrounding medium. By making subcultures from parts apparently free from bacterial growth he succeeded in obtaining the organism in the pure condition. At first the organisms were small, but after several days they had the usual length of the *Tr. pallidum* and all its characteristics. He stated that the organism multiplies by longitudinal division, but this is not generally accepted. Pure cultures may be grown in suitable (unheated) ascitic fluid, sufficient being placed in an $8 \times \frac{1}{2}$ inch test-tube to half fill it, to which is added a piece of fresh sterile rabbit's kidney, the whole then being covered by a half-inch layer of sterile vaseline (*Smith-Noguchi medium*). On inoculating monkeys (*Macacus* and *Cercopithecus*) by scarification, in some cases indurated syphilitic papules developed and the blood of the animals gave a positive Wassermann reaction. Other workers, however, have been unable to confirm these results on inoculating with cultures. Aksjanew-Malkin has recorded successful cultivation on the surface of an agar medium containing 25 per cent. of rabbits' blood by the use of Fortner's method (p. 96). Hoffmann and Frohn also reported recently obtaining a culture in rabbit

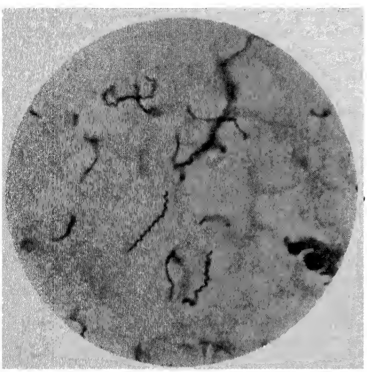


FIG. 159.—Section of brain in general paralysis showing a clump of *Treponema pallidum*. Jahnel's method. ×1000.

serum broth *plus* rabbit liver. This was derived from a closed lesion in a rabbit due to inoculation with the Nichols strain, and the fifth subculture proved virulent for a rabbit. It may be said that the cultivation of *Tr. pallidum* presents great difficulties, and the cultures obtained have usually been without pathogenic action. Accordingly, it is by no means generally admitted that the spirochætes hitherto obtained in cultures are genuine *Tr. pallidum*.

Transmission of the Disease to Animals.—Although various experiments had previously been made from time to time by different observers, in some cases with reported successful result, it is to the work of Metchnikoff and Roux (1903–05) that we owe most of our knowledge. These observers carried on a large series of observations, and showed that the disease can be transmitted to various species of monkey. Of these the anthropoid apes are most susceptible, the chimpanzee being the most suitable for experimental purposes. Their results have been confirmed by Lassar, Neisser, Kraus, and others. The number of experiments on these animals is now very great, and the general result is that the disease has been transmitted by material from all the kinds of syphilitic lesions in which spirochætes have been demonstrated, including tertiary lesions and the blood in secondary syphilis. Inoculation is usually made by scarification on the eyebrows or genitalia; but other parts of the skin are also susceptible in the higher apes. The subcutaneous and other methods of inoculation, with the exception of intratesticular and intravenous, give negative results. The primary lesion is in the form of an indurated papule or of papules, in every respect resembling the human lesion. Along with this there are marked enlargement and induration of the corresponding lymphatic glands. The primary lesion appears on an average about thirty days after inoculation, and secondary symptoms develop in rather more than half of the cases after a further period of rather longer duration. These are of the nature of squamous papules on the skin, mucous patches in the mouth, and sometimes palmar psoriasis. As a rule, the secondary manifestations are of a somewhat mild degree, and in no instance has any tertiary lesion been observed, though this may be due to the animals not having lived long enough. By re-inoculation from the lesions, the disease may be transferred to other animals. The disease may also be produced in *Macacus* and other lower monkeys, but these animals are less susceptible, and, moreover, the inoculation to be successful must be made at the special sites mentioned above; secondary manifestations do not appear. The severity of the affection amongst apes would in fact appear to be in pro-

portion to the nearness of the relationship of the animal to the human subject. The blood of the infected animals comes to give a positive Wassermann reaction. This may be the case even where no primary or other lesion develops, as in recent experiments of Schlossberger and Schmitz, who inoculated *Macacus rhesus* monkeys on the eyebrows or intra-testicularly, either with strains from the infection in rabbits or with fresh human syphilitic material.

As shown first by Hänsell, and afterwards by Bertarelli, the eye of the rabbit is susceptible to inoculation from syphilitic lesions. The material used is introduced in a finely divided state either into the tissue of the cornea or into the anterior chamber, and syphilitic keratitis or iritis, or both, may result, there being a period of incubation of at least two weeks. Levaditi and Yamanouchi have studied the stages in detail, and find that the spirochætes remain in the inoculated material unchanged for a time ; then organisation occurs and the spirochætes multiply, and later still there is a more rapid multiplication and invasion by them of the tissues of the eye. The period of incubation is thus not due to the organism passing through some cycle of development, but simply to its requiring certain conditions for multiplying which are not supplied for some time. The testis of this animal is also a convenient site of inoculation, a syphilitic orchitis being set up, and by this method the disease has been passed through long series of animals. Intra-testicular inoculation leads to general invasion of the body, and, as Brown and Pearce have shown, metastatic lesions occur, the development of which is favoured by certain circumstances, such as excision of the primary lesion. Spontaneous healing tends to occur after the infection has passed through a series of relapses, but even when all signs of the disease have disappeared virulent spirochætes are still present in the body, especially in the lymph glands. The intra-testicular method has proved of great value in testing the infectivity of suspected material, and by this means it has been shown that spirochætes from gummata are not attenuated in virulence. Often, however, the spirochætes in human syphilitic products show little virulence for the rabbit. Sometimes, where the primary inoculation has not led to a lesion, increase in virulence of the spirochætes for the rabbit has been attained by inoculating a second animal some weeks later with material from the popliteal lymph gland of the first. After several such passages a lesion containing spirochætes has developed in the inoculated testis. But only a few strains seem capable of propagation indefinitely in rabbits, *e.g.* those

of Nichols, Truffi, and several others. Uhlenhuth and Mulzer produced generalised syphilitic lesions in young rabbits by intra-cardiac inoculation with syphilitic material. They have also found that the organism can pass through the placenta of the rabbit and infect the fœtus. Further, the spirochætes are able to penetrate normal mucous membranes in the rabbit; but frequently no primary lesion develops, also the course of the resulting infection is mild (Brown and Pearce). According to Chesney and Kemp, a pre-existing lesion of the skin, such as an old granulating wound, affords in rabbits a very favourable site for the development of a primary chancre.

In the mouse *Tr. pallidum* produces no obvious effects, but if a small piece of rabbit's testis rich in spirochætes be implanted under the skin the organisms invade the tissues of the mouse, which, including the brain, are found still to be capable of infecting rabbits many months later (Schlossberger).

The experimental production of the disease has supplied us with some further facts regarding the nature of the organism. It has been shown repeatedly that the passage of fluid containing *Tr. pallidum* through bacterial filters deprives it completely of its infectivity. Hindle and Elford, by means of graded filters of collodion membrane, have found that while *Tr. pallidum* will pass through a filter which retains *B. prodigiosus*, nevertheless it is held back when the porosity is less than 0·4 μ. In other words, no evidence was obtained of the existence of a special filterable phase, significantly smaller than the diameter of the spirochæte. It is also readily destroyed by heat, a temperature of 51° C. being fatal. On the other hand, defibrinated blood containing *Tr. pallidum* has been found still to be infective after keeping for forty-eight hours at room temperature.

Immunity Phenomena.—It has long been accepted that a person suffering from syphilitic disease generally does not develop fresh lesions on reinoculation, and this was shown by experimental methods to hold in the artificially produced disease in the ape. But when, as the result of antisyphilitic treatment, the experimental infection in monkeys was sterilised, susceptibility to reinoculation soon developed. Similarly a considerable number of cases in the human subject have been observed where, after treatment with salvarsan, a second attack of the disease has been contracted, the inference being that the first attack had been completely cured. Recent work on rabbits, however, has indicated that this view may require modification. It has been shown that after intra-testicular inoculation treat-

ment short of effecting complete sterilisation of the infection may restore susceptibility to reinoculation, or "superinfection" as it is termed, provided that the treatment is begun when the infection is recent. On the other hand, when the infection has lasted for a considerable time, then even if it is sterilised by treatment, reinoculation in the ordinary way does not lead to the development of a fresh primary sore; nevertheless the spirochætes may in a proportion of such animals invade the tissues, which consequently become infective again for fresh animals. Spirochæticidal properties have not been demonstrated in the serum of the immune animals. Accordingly there appears to be here a form of tissue immunity which in the early stages depends on the persistence of the infection, but which when treatment is begun late remains effective even although the infection has been sterilised. Such immunity in the rabbit has been found to be more pronounced when the original infection and the reinoculation are made with the same strain than when different strains are used on the two occasions. After a primary inoculation of the rabbit's cornea, however, although dissemination occurs, it has been found possible to produce a fresh syphilitic lesion in other situations; apparently in this case the local effects of the primary lesion are not sufficiently intense to develop the tissue immunity.

With regard to the production of immunity, very little of a satisfactory nature has so far been established. It has been found that the organism after passage through a *Macacus* monkey produces a less severe disease in the chimpanzee than when derived directly from the human subject, inasmuch as secondary lesions do not follow; the organism would thus appear to have undergone a certain amount of attenuation in the tissues of that monkey. But accidental infection of the human subject has occurred with spirochætes which had been repeatedly passed through rabbits during a period of over a year and a half. The presence of the spirochæte does not lead to the formation of specific antibodies to any marked extent.

Luetin.—Noguchi prepared an extract from pure cultures of the *Treponema pallidum*, which he called *luetin*, and he found that this gave a characteristic cutaneous reaction in syphilitics, which is analogous to the tuberculin reaction in tuberculosis (p. 433). Although the reaction may be of value for detecting latent syphilitic infection, it appears that it is not specific in the etiological sense. In positively reacting subjects other substances, *e.g.* agar or killed cultures of bacteria, may produce a similar reaction. Again, non-syphilitic individuals come to react positively as a result of the administration of iodides (Sherrick).

Serum Diagnosis—Wassermann Reaction and Flocculation Reaction.—The method of applying the Wassermann test has already been given (p. 152); we have now to consider the results of its application. It will not be an overestimate to say that a positive result may be obtained in at least 90 per cent. of cases where there is evidence of active general infection. The reaction generally appears first on the tenth to thirtieth day after appearance of the sore, and then gradually becomes more marked; during the period of secondary manifestations it is practically always present; in the tertiary stage with active manifestations a positive result is only a little less frequent. In cases of congenital syphilis with active lesions the proportion of positive reactions is nearly as great as in the secondary stage of the acquired disease. As the disease becomes inactive or is cured the reaction may disappear, but it is to be noted that disappearance of the reaction after being present does not necessarily imply cure of the disease. It may only have become latent, and on its becoming once more active the reaction may reappear; in fact, its presence would appear to be definitely related to the activity of the syphilitic lesions. A positive reaction is practically always present in general paralysis and in the large majority of cases of tabes, and is as frequently given by the cerebro-spinal fluid as by the blood serum in these diseases; in certain cases of neurosyphilis the cerebro-spinal fluid may react positively when the blood serum is negative. As regards other diseases, a positive reaction has been recorded as occurring in leprosy (p. 447) and sleeping-sickness and also in yaws and bezel, and occasionally in malaria during the febrile periods; but apart from these diseases it is practically never met with. At present little can be said in explanation of the Wassermann reaction. It seems to depend on the interaction of lipoidal substances in the extract with proteins in the serum, which are apparently contained in the globulin fraction. It is now accepted that it does not depend on the presence of an immune-body which in association with the causal agent (the spirochæte) fixes complement. Although there is considerable evidence that the reacting power of the serum is due to an antibody for certain lipoids (see Dunlop and Sugden), we do not know why this should be present in syphilis. The various forms of flocculation reactions (Sachs and Georgi, Meinicke, "Sigma" of Dreyer and Ward, Kahn, "Citochol," etc.) give results which are comparable with those of the Wassermann test. A small proportion of sera from cases with a history of syphilis react positively in the flocculation test but negatively in the Wassermann reaction,

while occasionally the converse occurs. Thus neither the Wassermann test nor the flocculation test alone will detect all syphilitic sera ; but when a series of sera is examined both yield practically the same number of positive results. The most complete information is obtained when the two tests are used in conjunction, the one serving as a confirmation of the other, especially when a weakly positive result occurs. Although the technique of the various flocculation methods is relatively simple, experience in reading the results is required.

Methods of Examination.—As already said, in the examination of an ulcerated chancre or other lesion it is advisable to get rid of the surface organisms. The surface should be cleansed with saline and dried. A piece of cotton wool soaked in absolute alcohol or spirit is then applied for about a minute ; the alcohol is then washed off with saline, and the surface is again dried. After a short time there is usually a free flow of watery serum, which is practically free from other organisms, and often contains the spirochæte in large numbers ; a small drop of this is placed on a slide, a cover-glass is applied, and the specimen is examined by dark-ground illumination. It is advisable to put a thin ring of vaseline on the slide to support the cover-glass. When examination must be delayed some of the serous exudate is allowed to flow into a capillary tube ; this is then sealed. The spirochætes under these conditions retain their shape for days or weeks. Dried films also may be made and treated by any of the methods above described (p. 128) ; the modification of Becker's is recommended. Others prefer to scarify the margin of the sore and examine the lymph which exudes, the flow of which may be aided by squeezing, or a small incision may be made with a very sharp knife, and then after bleeding has completely stopped to take the small drop of serum which gathers at the site. In all cases admixture of blood is to be avoided, as it interferes with the examination by the dark-ground method. It is important that neither local antiseptic applications nor any form of antisyphilitic treatment should be given before the examination is made. In the case of a lymphatic gland or non-ulcerated lesion it is best to puncture with a hypodermic needle, the point of which should be moved about in the tissue. After it is withdrawn a little saline may be placed in the syringe and pressed through the needle, the first small drop which passes, and which washes out the contents, being taken for examination ; here also dark-ground illumination gives the best results. Tissues are fixed in 5 per cent. formalin and treated by Levaditi's or Jahnel's methods (p. 126). An emulsion of tissue in saline may also be examined by dark-ground illumination.

Tr. cuniculi.—This organism, which is indistinguishable microscopically from *Tr. pallidum,* is found in naturally occurring chronic ulcers in the neighbourhood of the mouth and genitalia in rabbits. The infection can be transmitted experimentally by scarification and inoculation with material from the ulcers, but only rabbits (not man or other animals) are susceptible. The organisms proliferate abundantly in the deep layers of the epidermis, but they

have little tendency to invade the underlying tissues or to spread to the internal organs.

Treponema Pertenue

Frambœsia or yaws is a disease of the tropics, occurring in the west coast of Africa, Ceylon, the West Indies, and other parts. It is characterised by a peculiar cutaneous eruption, and it is markedly contagious. Its resemblance in many respects to syphilis has been noted, and the relation of the two diseases has been the subject of much controversy. It is accordingly a matter of great interest that an organism of closely similar characters to the *Treponema pallidum* has been found in the lesions of frambœsia. This organism was discovered by Castellani, who gave to it the name *Spirochæta pertenuis* or *pallidula* (*Treponema pertenue*, Castellani). Morphologically, it is practically identical with the *Treponema pallidum* (Fig. 160); when ulceration has occurred various spirochætes of other types may be present as contaminants. In the skin lesions it has been shown by Levaditi's method to be present in considerable numbers, especially in the epidermis and also amongst the leucocytic infiltration, which comprises more polymorphonuclear leucocytes than are seen in the case of syphilis. Castellani showed that the disease could be transferred to monkeys (*Semnopithecus* and *Macacus* being used for this purpose), and that the organism could be demonstrated in the unbroken skin lesions. The lesions are as a rule confined to the site of inoculation, but the infection is general, as is shown by the presence of spirochætes in the lymphatic glands and the spleen, although they do not survive in the lymphatic tissues after the skin lesions have

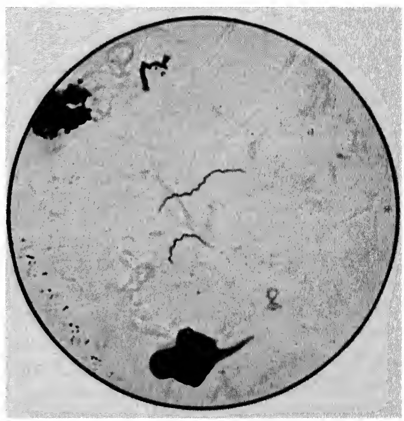


Fig. 160.—*Treponema pertenue*. Film preparation from skin lesion in yaws, stained by Becker's method. ×1000.

disappeared and in this respect differ from *Tr. pallidum* (Schöbl). These results with regard to the presence of *Treponema pertenue* in the lesions, and the inoculation of apes have been confirmed by other workers, and the etiological relationship of the organism to the disease may now be regarded as established. Nichols has shown that a frambœsia lesion can be produced in the testicle of the rabbit of similar character to the syphilitic lesion, though the period of incubation is shorter. He finds that the best means of distinguishing the two diseases is afforded by inoculating the skin of the monkey. In the case of syphilis the resulting lesion is flat, dry, and very scaly; in the case of frambœsia it is elevated, slightly scaly, and very œdematous; here also the period of incubation is shorter in the case of frambœsia. Pearce and Brown observed on intra-testicular inoculation with *Tr. pertenue* in the rabbit a granular or finely nodular peri-orchitis—a type of lesion quite different from that observed in the case of *Tr. pallidum*. Similarly, Turner and Chesney working with a number of strains of *Tr. pertenue* isolated from cases in Haiti, found that in rabbits the characteristic initial testicular lesion consisted of scattered miliary granules in the substance of the organ, the tunica or the epididymis. The great enlargement or marked induration, so common after inoculation with *Tr. pallidum*, did not occur. On intracutaneous inoculation, again, a superficial lesion without induration resulted, in contrast to the raised indurated button-like chancre due to *Tr. pallidum*. It is noteworthy that a strain of the syphilitic spirochæte isolated in Haiti at the same time as the frambœsia strains, showed the behaviour in rabbits which is characteristic of other strains of *Tr. pallidum*. Further, in frambœsia in rabbits there is not the tendency to development of generalised lesions which is characteristic of syphilis. In mice, Schlossberger found that the spirochæte of frambœsia behaves like that of syphilis (p. 675).

The immunity reactions in monkeys and rabbits infected with syphilis and frambœsia have been experimentally studied with contradictory results. According to the thorough investigations of Schöbl and Miyao, monkeys which had recovered from yaws and were immune to reinoculation with *Tr. pertenue* developed only very slight lesions on inoculation with a strain of *Tr. pallidum* which was virulent for the rabbit and for normal monkeys. Further, the syphilitic spirochætes were not able to persist in the tissues of monkeys immune to frambœsia, as was shown by the non-infectivity of the lymph glands of the latter for rabbits.

Schöbl has also recorded observations regarding the influence exerted by inoculation with *Tr. pallidum* on a pre-existing latent infection with *Tr. pertenue* in monkeys. Animals which had been injected with killed *Tr. pertenue* developed no local lesion on repeated inoculation of the skin with these organisms in the living state. Later, after an inoculation with syphilitic spirochætes no lesion developed at the site, but after about three months or longer a typical yaws lesion appeared at the site last inoculated with *Tr pertenue*. Thus apparently infection with *Tr. pallidum* can increase the reactivity of the tissues to the yaws spirochæte.

Therefore it is not possible to decide by such methods whether frambœsia and syphilis are due to distinct although related organisms, or whether frambœsia is merely a modified form of syphilis. We may add that patients suffering from frambœsia generally give a positive Wassermann reaction ; they are also very amenable to treatment with salvarsan (Alston and others). The exact relationship of the two diseases cannot be yet accurately defined, but they are probably distinct, though undoubtedly closely related.

Bezel

A disease intermediate in its clinical characters between yaws and syphilis, known as " bezel," and relatively mild in its effects, occurs in Arabs of the Euphrates region (Hudson) ; it affects children chiefly. As there is no initial lesion, the site of entry of the infection is not clear, but sexual contact plays little part in its transmission. The first sign of the disease is an eruption in the mouth and then on the skin. Spirochætes resembling *Tr. pallidum* are readily found in the lesions and the Wassermann reaction is usually positive. So far information as to experimental transmission to animals is not available.

CHAPTER XXII

SPIROCHÆTES OF THE RELAPSING FEVERS: LEPTOSPIRA ICTEROHÆMORRHAGIÆ, ETC.: SPIRILLUM MINUS

SPIROCHÆTES OF THE RELAPSING FEVERS

AT a comparatively early date, namely, in 1873, when practically nothing was known with regard to the production of disease by bacteria, a highly characteristic organism was discovered by Obermeier in the blood of patients suffering from relapsing fever. This organism is now known as the *Treponema* or *Spirochæta obermeieri* (or *Borrelia recurrentis*). Obermeier described its microscopical characters, and found that its presence in the blood had a definite relation to the time of the fever, as the organism rapidly disappeared about the time of the crisis, and reappeared when a relapse occurred. His observations were fully confirmed, and his views as to its being the cause of the disease have been established.

Relapsing fever as it occurs in different parts of the world has now been carefully studied, and the relationships of the organisms have been the subject of much investigation and discussion. It has been proved that all these infections are caused by spirochætes and are transmitted by blood-sucking arthropod vectors—the European, North American, Asiatic, and North African infections by the body louse; the Central African, Persian, Central and Southern American types by a tick, as originally suggested by the designation "tick fever." It has also been shown that spirochætal diseases are widespread amongst vertebrates; they have been described, for example, in geese by Sacharoff, in fowls by Marchoux and Salimbeni, in oxen and sheep by Theiler, and in bats by Nicolle and Comte, and it is interesting to note that in the case of the spirochætoses of oxen and fowls the infection is transmitted by means of ticks.

Spirochæta obermeieri (*Borrelia recurrentis*)—**Microscopical Characters.**—This spirochæte will be described in detail, since

it was the one first discovered; but the spirochætes of all the forms of relapsing fever are alike in their microscopical characters. The organisms as seen in the blood during the fever are delicate spiral filaments which have a length of from 10 to 20 μ, but even longer forms occur; their thickness is about 0·35–0·5 μ. They show several fairly regular coils about 2–3 μ in length and varying in number according to the length of the organisms; their extremities are pointed (Figs. 161, 162) They are actively motile, and may be seen moving across the microscope field with a peculiar movement which is partly rotatory and partly undulatory, and disturbing the blood corpuscles in their course. Multiplication takes place by transverse division. It has been stated that they are capable of passing through filters which retain the ordinary bacteria; this is probably due to their flexibility. The most satisfactory method of demonstrating the organisms is by dark-ground illumination[1]; very rarely strains are met with which show a double contour. They stain with watery solutions of the basic aniline dyes, though somewhat faintly. They are best coloured by the Romanowsky stain or one of its modifications and then usually have a uniform appearance throughout, or may be slightly granular at places. They are Gram-negative. There is no evidence that they form spores. Swellings observed in the organisms have been variously interpreted either as degenerations or as a phase in development ("chromatin granules"). In dry-fixed preparations the windings are irregular. The significance of coiled-up forms of the spirochæte has not been determined.

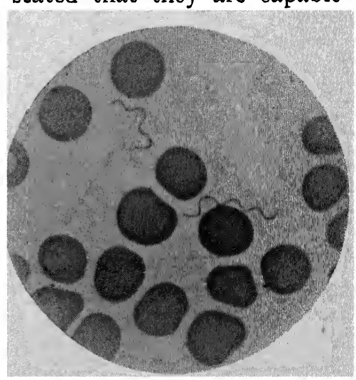


FIG. 161. — Spirochætes of relapsing fever in human blood (case from India). Leishman's stain. ×1000. See also Plate IV., Fig. 19.

Cultivation.—Levaditi observed multiplication of *Sp. duttoni* (*vide* p. 690) in cultures made by inoculating heated serum in collodion sacs, which were then closed and placed in the peri-

[1] In examining blood by the dark-ground method, it is important that the observer should be familiar with "pseudo-spirochæte" artefacts which are derived from the red corpuscles. They show Brownian movement but not progression.

toneal cavity of rabbits; after repeated subcultures the organisms were still infective for animals. Novy found that the spirochæte of American relapsing fever remained alive and virulent in defibrinated rat's blood for forty days. Noguchi succeeded in cultivating the spirochætes of the various relapsing fevers by the following method. A piece of sterile tissue, *e.g.* kidney of rabbit, is placed in a test-tube; a few drops of citrated blood from an infected animal are added and then 15 c.c. of sterile ascitic or hydrocele fluid. The presence of a loose fibrin coagulum seems to favour growth. It should be noted that the spirochæte is not strictly anaerobic, although it flourishes

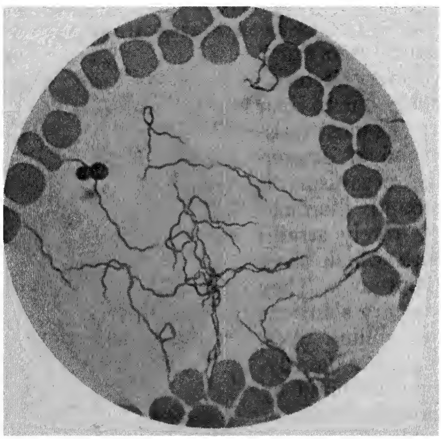


FIG. 162.—*Spirochæta obermeieri* in blood of infected mouse. Leishman's stain. ×1000.

best at a reduced oxygen tension. In cultures after a few days the spirochætes tend to degenerate and disappear, but re-growth may occur. In order to maintain virulence in cultures it has been found advisable to subculture at the height of the original growth (fourth day).

Cultures have also been obtained by Kligler and Robertson in a medium of horse or rabbit serum diluted with 1 to 2 parts of saline, or of ascitic fluid undiluted. To 10 parts of the fluid used 1 part 10 per cent. peptone broth is added, and the reaction set to pH 7·2–7·4. A drop of blood from an infected animal is used to inoculate 3–4 c.c. of the medium in a tube of 1 cm. diameter, which is then covered with a thin layer of liquid paraffin. In the case of subcultures a drop of rabbit's blood is added to the medium to supply fibrin (*vide supra*). The cultures are incubated at 28° to 32° C. Another medium which has been advocated is 20 per cent.

rabbit serum and 80 per cent. Hartley's broth in tubes containing 1 gram coagulated egg albumin . this medium also is covered with a paraffin seal (Lapidari and Sparrow) Galloway's method is simple ; it consists in placing a small amount of egg-white in a test tube which is kept slanting in a hot-water bath, so that the coagulum may have as large a surface as possible ; then 5 c c. of a dilution of serum which has previously been heated for one hour at 58°–60° C. is added (rabbit's serum is diluted 1 : 5, horse serum 1 · 10) and the mixture is covered with sterile melted vaseline Sterility is controlled by keeping the tubes at 37° C. for twenty-four hours before inoculation. The addition of a drop of fresh human or rabbit blood favours growth.

Relations to the Disease.—In relapsing fever, after a period of incubation there occurs a rapid rise of temperature which lasts for about five to seven days. At the end of this time a crisis occurs, the temperature falling quickly to normal. About seven days later a sharp rise of temperature again takes place, but on this occasion the fever lasts a shorter time, again suddenly disappearing. A second or third relapse may occur after a similar interval. The organisms begin to appear in the blood shortly before the onset of the pyrexia, and during the rise of temperature rapidly increase in number. They are sometimes very numerous during the fever, a large number being often present in every field of the microscope when the blood is examined at this stage. In some cases, however, they may be less numerous and even difficult to find by microscopic examination (this is especially true of tick-fever). They begin to disappear shortly before the crisis ; during the afebrile interval they usually cannot be found in the circulating blood on microscopic examination, but the blood is as a rule still infective. A similar relation between the presence of the organisms in the blood and the fever is found in the relapses ; but here the organisms tend to be less numerous than in the first attack. Münch in 1876 produced the disease in the human subject by injecting blood containing the spirochætes, and this has been repeatedly confirmed. Recently inoculation has been practised as a therapeutic measure in general paralytics (cf. p. 805). Carter in 1879 was the first to show that the disease could be readily produced in monkeys, and his experiments were confirmed by Koch. In such experiments the blood taken from patients and containing the spirochætes was injected subcutaneously. In the disease thus produced there is an incubation period which usually lasts about three days. At the end of that time the organisms rapidly appear in the blood, and shortly afterwards the temperature quickly rises. The period of pyrexia usually lasts for two or three days, and is followed by a marked

crisis. As a rule there is no relapse, but occasionally one of short duration occurs. Norris, Pappenheimer, and Flournoy, in their experiments on monkeys with the organism of American relapsing fever, found that several relapses occurred. Cunningham, working with strains from Indian relapsing fever, found that in monkeys a relapse frequently occurred. He points out that in animals relapses are often transient and may easily be missed. White mice and rats are also susceptible to infection, and after repeated passages in these animals relapses regularly occur; but when the infection has been recently derived from man there is often only a single attack with disappearance of the organisms by crisis. In mice the infection tends to persist in a latent state in the brain. Various other rodents are susceptible to inoculation. Cunningham in India has used squirrels; these, after inoculation, develop a primary infection which is frequently followed by a single relapse, as in the human disease, a second relapse being rare. When relapsing fever is contracted during pregnancy the fœtus may become infected.

Immunity.—Metchnikoff found that during the fever the spirochætes were practically never taken up by the leucocytes in the circulating blood, but that at the time of the crisis, on disappearing from the blood, they accumulated in the spleen and were ingested in large numbers by the macrophages and polymorphonuclear leucocytes. Within these they rapidly underwent degeneration and disappeared. It is to be noted in this connection that enlargement of the spleen is a very marked feature in relapsing fever. That the spleen exercises a special function in the resistance to relapsing fever appears clearly from the experiments of Meleney; he found that in order to obtain a relapsing infection in squirrels with a Chinese strain of the spirochæte, it was necessary to inoculate animals whose spleens had previously been removed. This property of the spleen has been related to its large content in cells belonging to the reticulo-endothelial system. As in the case of so many other diseases, however, an all-important factor in the destruction of the organisms is the development of antagonistic substances in the blood. Lamb showed in the case of the monkey (*Macacus radiatus*) that the immunity following an attack of the disease was due to the presence of spirochæticidal bodies in the serum. He found, for example, that *in vitro* the serum of an immune animal brought the movements of the spirochætes to an end, clumped them, and caused their disintegration; and further, that in one case when the spirochætes and the immune serum

were injected into a fresh monkey, no disease developed. Observations by Sawtschenko and Milkich, Novy and Knapp, and Rabinowitsch, also showed that in the course of infection there are developed anti-substances of the nature of immune-bodies, with protective properties, and agglutinins. Brussin showed that with these organisms an immunity phenomenon can be demonstrated similar to that with trypanosomes (p. 837); when antibody-containing blood is added to the homologous spirochætes the latter become coated with blood platelets, or if antiserum and a culture of bacteria, e.g. *B. coli*, are mixed with the spirochætes the latter become coated with the bacteria —the adhesion phenomenon. It is best seen by dark-ground examination. Novy and Knapp produced a " hyper-immunity " in rats by repeated injections of blood containing the spirochætes, and found that the serum of such animals had a markedly curative effect, and could cut short the disease in rats, mice, and monkeys. The course of events in the human disease has been explained on the basis of a transient immunity developing during the first period of pyrexia, some of the spirochætes, however, in internal organs or in tissues escaping destruction by the serum or by phagocytes. These organisms are then able to multiply and reappear in the blood, possibly in part as a result of disappearance of the immunity, but also because a strain has developed which is resistant to the protective anti-bodies (Levaditi and Roché). In the case of Indian relapsing fever, in which there is usually only one relapse, Cunningham and others have shown that the strain of spirochæte present in the relapse is serologically distinct (as judged by agglutination reactions) from that observed in the original attack, and the same holds good for experimental infections. Further, experimental infection with one of these two serological types is followed by a relapse due to the other, the types present in the original attack and in the relapse alternating regularly; but where repeated relapses occur additional antigenic types may appear or there may be reversion to an earlier type. These observations suggest that the antigenic character of the organism undergoes alteration by the influence of the defences of the body, and offer a further explanation of the relapsing nature of the infection. The immunity following an attack in man disappears within several years. The immunity reactions above described depend on a very delicate adjustment between the antibody and the parasite. Thus passage through a different host, as well as the influence of the latter at different stages of the infection, tend to modify the antigenic properties of the spirochætes. Accord-

ingly, these reactions have little value for the identification of different species.

Varieties.—As already stated, relapsing fever has been studied in different parts of the world and differences have been made out with regard to clinical features, pathogenic effects in man and on experimental inoculation, arthropod vectors and immunity reactions. It has been emphasised by Brumpt that points of special importance in distinguishing the different forms of relapsing fever are the species of arthropod which is capable of acting as the transmitter, and the susceptibility of experimental animals to infection, especially the guinea-pig. The latter resists infection with louse-borne spirochætes but may be infected with some of those which are tick-borne. Apart from tick fever, European, Asiatic, and North American types have been distinguished, all of which are transmitted by lice (*vide infra*). Relapsing fever in Asia is apparently a much more severe disease than in Europe; F. P. Mackie gives the mortality in Bombay at the high figure of 38 per cent., but differences in this respect, as well as in pathogenic effects, may depend on variations in virulence of the spirochæte or on the susceptibility of the population attacked. The spirochæte of Indian relapsing fever has been called *Sp. carteri*, that of the North American infection *Sp. novyi*, while the designation *Sp. obermeieri* may be retained for the European variety. Sergent and Foley have described a type of relapsing fever in Algiers and have given the name *Sp. berbera* to the organism concerned. It has been concluded from the work of Novy, Strong, and F. P. Mackie that the American spirochæte is probably a distinct species, as animals immunised against it are still susceptible to infection with the European and Asiatic organisms and *vice versa*. The relation between the two latter is certainly closer and no distinct immunological differences have been established. But the limited value of such observations has been indicated above. Further, a form of relapsing fever similar to the European and Asiatic types and also transmitted by lice, occurs in West Africa. Accordingly, there is no strong evidence that the louse transmits more than one species of relapsing fever spirochæte. On the other hand, the spirochætes transmitted by ticks are markedly different in their properties from the above. Further, it is likely that several distinct species are transmitted by ticks.

Louse Transmission of Relapsing Fever.—The fact that African tick fever and certain other spirochætoses were known to be conveyed by the bites of arthropods suggested that

European, Asiatic, and North American relapsing fever is transmitted in this way. At first the bed-bug was believed to be the vector of transmission, but attempts to transmit the disease by means of the bites of bugs were generally unsuccessful; F. P. Mackie produced the disease in only one out of six monkeys used for this purpose, though large numbers of bugs, which had bitten relapsing fever patients, were used. On investigating an epidemic of the disease, however, he obtained a considerable amount of evidence on epidemiological grounds that the disease was carried by the body louse. This has been established by the work of Nicolle, Blaizot and Conseil, and by the experiments of Manteufel, who was able to transmit infection from rat to rat in nearly 60 per cent. of the experiments made, whereas he obtained only negative results by means of bugs. The mode of inoculation of the organism from infected lice is by its introduction into abrasions produced by scratching — the crushed insect providing the inoculum. Infection may also occur through intact mucous membranes. The spirochætes can be observed in the stomach of the louse for a day after infective blood has been ingested; after about six days they can be demonstrated in the body cavity, and then spread throughout the insect's body. It has been supposed that the organisms may be infective when in a non-spirochætal stage (Sergent and Foley).

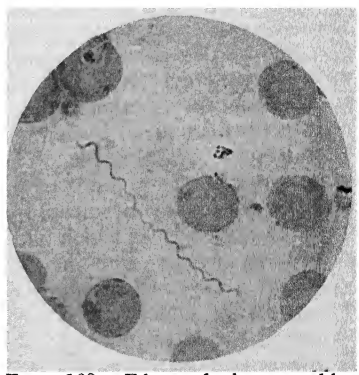


FIG. 163.—Film of human blood containing spirochæte of tick-fever, *Sp. duttoni*. ×1000.[1]

Tick Transmission.—Spirochætes had been seen in the blood of patients with tick-fever in Uganda by Greig and Nabarro in 1903, and Milne and Ross in 1904 recorded a series of observations which led them to the conclusion that tick-fever was due to a spirochæte. It is, however, chiefly owing to the work of Dutton and Todd in the Congo Free State, on the one hand, and of Koch in German East Africa, on the other, that our knowledge of the etiology of the disease has been obtained. Thus the

[1] We are indebted to the late Lt.-Gen. Sir William Leishman. A.M.S., for the preparation from which Fig. 163 was taken.

disease long known by the name of Tick-Fever as prevalent in Tropical Africa has been shown to be caused by a spirochæte—*Sp. duttoni* (*Sp. crociduræ*) (Fig. 163).

The following are the chief facts regarding this disease. Clinically, the fever closely resembles relapsing fever, but the periods of fever are somewhat shorter, rarely lasting for more than two or three days, and the relapses are more numerous. The organisms in the blood are considerably fewer than in the case of European relapsing fever, and sometimes they can only be demonstrated after prolonged microscopic search—in such cases a "thick" drop should be used as in searching for scanty malarial parasites (p. 809)—or by inoculation of a susceptible animal with the patient's blood. Dutton and Todd showed that it was possible to transmit the disease to certain monkeys (*Cercopitheci*) by means of ticks which had been allowed to bite patients suffering from the disease, the symptoms in these animals appearing about five days after inoculation. The disease thus produced is characterised by several relapses, and often leads to a fatal result. Ticks can be infected at any stage of development as a result of biting infected persons. In one case the disease was produced by means of young ticks hatched from the eggs of ticks which had been allowed to suck the blood of infected patients, and they came to the conclusion that the spirochætes were not simply carried mechanically by the ticks, but probably underwent some cycle of development in the tissues of the latter. Leishman showed that the ticks of the second generation may also be infectious. The species of tick concerned are chiefly *Ornithodorus moubata* and *O. savignyi* and sometimes *O. erraticus*. Koch found that after the ticks had been allowed to suck the blood containing the organisms, these could be found for a day or two in the stomach of the tick. After this time they gradually disappeared from the stomach, but were detected in large numbers in the ovaries of the female ticks, where they sometimes formed felted masses. He also traced the presence of the spirochætes in the eggs laid by the infected ticks, and in the young embryos hatched from them. On the other hand, Leishman failed to find evidence of spirochætes either in the tissues of ticks later than ten days after ingestion of infected blood, or in the ova laid by the ticks, or in the young ticks when hatched, though these were proved by experiment to be infective. After ingestion of the blood by the ticks, he found that morphological changes occur in the spirochætes, resulting in the formation of minute "chromatin granules" which are extruded and traverse the walls of the

intestine and are taken up by the cells of the Malpighian tubules; they also penetrate the ovaries and may be found in large numbers within the ova. Similar granules are to be seen in the Malpighian tubules of the embryo ticks, where they are also found in the subsequent stages of their life. He proved that infection of animals might be produced by inoculation with crushed material containing the granules but no spirochætes. He accordingly considered that the granules in question represent a phase in the life-history of the parasite, and that infection occurs by inoculation of the skin with the chromatin granules voided in the coxal secretion and not by unaltered spirochætes from the salivary glands. A similar view is taken by Hindle, who found that when infected ticks, in which the spirochætes had disappeared, were warmed to a temperature of 35° C., the spirochætes reappeared in the organs and cœlomic fluid. These "metacyclic" forms differ from those in the blood in being thinner, shorter, more irregular and less deeply staining.

Koch also made extensive observations on the ticks in German East Africa, and found that of over six hundred examined along the main caravan routes, 11 per cent. contained spirochætes, and in some localities almost half of the ticks were infected. In places removed from the main lines of commerce he still found them, though in smaller number. It has also been demonstrated that in some places the ticks are found to be infected with the spirochætes although the inhabitants do not suffer from tick fever, a circumstance which is possibly due to their having acquired immunity against the disease.

It is now generally believed that the *Sp. duttoni* is a species distinct from, though closely allied to, the organisms of the relapsing fevers, described above. We have mentioned differences in the clinical characters of the diseases, and there are also differences in the pathogenic effects of the organisms on inoculation. The *Sp. duttoni*, for example, produces a much more severe disease in monkeys, and is pathogenic to more species of the laboratory animals than the *Sp. obermeieri*. Experiments have indicated also that there is a specific relation between each parasite and its invertebrate host; the tick-borne infection is not transmissable by lice. Differences brought out by immunity reactions both *in vivo* and *in vitro* have also been adduced as evidence by Breinl and others that these spirochætes belong to distinct species. As has been stated already, however, the variability which a given strain of spirochætes may exhibit renders the method of doubtful value.

Tick-borne relapsing fever also occurs in Spain, Central and South America, Arabia and Persia. The Spanish form, due to *Sp. hispanica,* is a mild disease ; it is transmitted by *O. erraticus.* In Central America sporadic infections occur, due to *Sp. novyi* and *Sp. turicatæ.* The latter has been shown by Brumpt to be transmitted by *O. turicata* in Texas and by *O. hermsi* in California. It is of importance that small rodents naturally harbour this spirochæte and so may maintain the infection in the ticks which infest them. In South America *Sp. venezuelensis* is transmitted by *O. venezuelensis* and *O. talaje. Sp. persica* (of the Persian disease) appears to be transmitted directly by the bite of *O. papillipes,* since it does not excrete coxal fluid or fæces while biting.

Leptospira Icterohæmorrhagiæ

Spirochætal or infective jaundice, long known as Weil's disease, was proved in 1915 by Inada and other Japanese workers to be due to a spirochæte, to which they gave the name *Spirochæta icterohæmorrhagiæ* (*Leptospira icterohæmorrhagiæ*). This spirochæte is characterised by its exceedingly numerous and fine closely wound coils, and in 1917 Noguchi applied to it the generic name of *Leptospira* on account of its morphology. The pathology of the condition has now been extensively studied. The disease is characterised by the sudden onset of symptoms, general malaise, pyrexia lasting about ten days, a tendency to hæmorrhage from mucous surfaces, conjunctival congestion, hæmorrhagic herpes, and jaundice which becomes increasingly marked from the fourth day of the illness. Meningitis has been recorded in this infection. The occurrence of the disease in small epidemics had been previously noted. In Japan it was found to occur among workers in the same part of wet mines. During the war the troops in France were affected and the results of the Japanese workers were confirmed by bacteriologists in both the British and French armies. It was also found on the Italian front and among the German troops. The mortality was much lower than that met with in Japan. On the Continent the disease has in recent times been shown to have a wide endemic distribution. It has been described in Scotland by Gulland and Buchanan among coal miners working in wet mines, and by Davidson and others among fish workers. It has also occurred in sewer workers in London (Fairley). In Holland there have been frequent cases, especially following immersion in canal water.

Morphology of the Spirochæte.—The organism in the blood and tissues measures 6–9 μ in length (but both shorter and longer forms occur) and about 0·25 μ in thickness; that is, it is a slender organism of about the thickness of the *Tr. pallidum*. In cultures it may grow into much longer forms. It is somewhat thicker in the middle and tapers towards the ends, which may be pointed, but there are no terminal flagella (Figs. 164, 165). The morphology is best demonstrated by dark-ground illumination and is characterised by numerous fine "elementary" spirals and recurved or "hooked" ends, while occasionally secondary spirals or undulations are observed (Fig. 166). In fixed and stained preparations the elementary spirals are not easily demonstrable (Fig. 164). It is actively motile, the movements being rotatory and undulatory. It can be studied by all the microscopic methods already described in the case of the *Tr. pallidum* (p. 667).

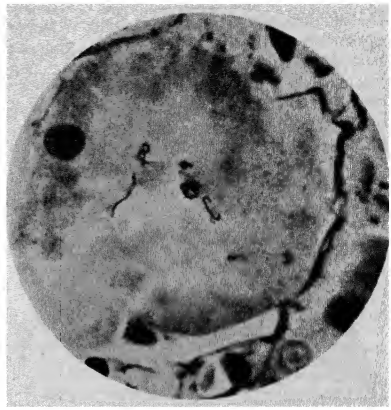


FIG. 164.—*Leptospira icterohæmorrhagiæ* in a renal tubule of a wild rat; section stained by Levaditi's method. Note the typical hooked ends of one of the spirochætes. ×1000 (approx.).

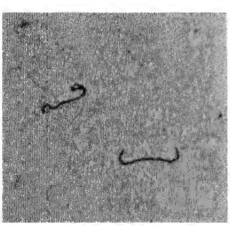


FIG. 165. — *Leptospira icterohæmorrhagiæ*, from a culture in Noguchi's medium. Stained by Kirkpatrick's method (p. 124.) ×1000.

Cultivation. — The organism was first successfully cultivated in Noguchi's medium for *Tr. pallidum*, in which the initial growth survived for three to six weeks. Later it was grown in solid media, blood agar and blood gelatin, the former being the more suitable. The limits of growth are wide, namely, 13°–37° C., the optimum temperature being 25°–30° C. The following media are suitable:

Noguchi's Medium.—(*a*) A mixture of rabbit's serum, 2 parts, Ringer's solution (or 0·9 per cent. NaCl solution), 6 parts, citrated

rabbit's plasma (or sterile defibrinated rabbit's blood), 1 part. (*b*) The same with the addition of 1–2 parts of neutral or slightly alkaline 2 per cent. agar, which should be melted and added when at a temperature of 60°–65° C. in order to get a uniform mixture. Both media are covered with a layer of sterile liquid paraffin, and inoculation is made through the paraffin. The above are most suitable for primary cultures. For subcultures one may use Wenyon's modification prepared as follows: to 270 c.c. of 0·85 per cent. sodium chloride solution (pH 7·6) are added 30 c.c. of ordinary 2 per cent. bacteriological nutrient agar (pH 7·6). When mixture has taken place, 10 c.c. are placed in each test-tube. After autoclaving at 120° C. the tubes are cooled to 50° C. and into each tube are allowed to drop, from a rabbit's ear, 20 drops of blood under aseptic precautions (see p. 68). The tubes are not shaken or mixed, and are incubated for twenty-four hours at 37° C. The medium is then ready for use. In such media growth produces a visible opacity in the top layer. Most abundant growth is obtained when medium is disposed as a thin layer in flat-bottomed flasks.

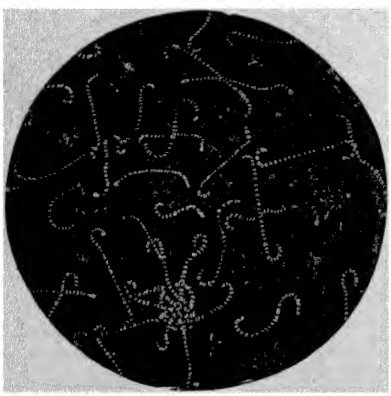


FIG. 166.—*L. icterohæmorrhagiæ*, as seen in a culture by dark-ground illumination, showing elementary spirals. (From a drawing by Dr. Geo. Buchanan.)

Fletcher's medium has been found by Taylor and Goyle to yield primary cultures with great regularity in early cases and to maintain them alive for long periods. It is prepared as follows: Pooled serum from several rabbits is added to sterile distilled water in a flask to yield a 10 to 12 per cent. serum-dilution. The serum-water mixture is heated to 50° C. in a water bath and to each 100 c.c., 6 c.c. of melted nutrient 2·5 to 3 per cent. agar, pH 7·5, are added and well mixed. There should be no flocculent particles visible to the naked eye. The reaction is adjusted to pH 7·4 and the medium is tubed in 5 c.c. quantities and heated at 56° C. for one hour on two successive days. A similar medium without the agar may be more suitable for agglutination tests, but in it growth is not so profuse.

Relation to the Disease.—The organism occurs both in the blood and in the organs. In the former it is found in the first five or six days of the disease; thereafter it gradually disappears, and in the second week, when jaundice is most marked, it cannot be detected. The best method of demonstrating its presence is to draw off some blood, say 3–5 c.c., and inject it into

the peritoneal cavity of the guinea-pig, in which animal it produces an infection and can easily be found (*vide infra*); it is of importance that the leptospira may survive for some days in clotted human blood. It is rarely present in the blood in the human subject in numbers sufficient to allow its detection by direct microscopic examination; according to Kligler and Ashner, a satisfactory method is to centrifuge the citrated blood at 1000–1500 revolutions per minute for five to ten minutes, then to separate the supernatant fluid and to centrifuge the latter at higher speed (3000 revolutions) for two hours and to examine the resulting sediment. Taylor and Goyle in the Andamans found blood culture to be the best method of diagnosis when carried out between the third to the sixth day of illness; in each case 4 or 5 tubes of medium, *e.g.* Noguchi's, were inoculated with 0·1–1 c.c. blood and kept seven to twenty-one days (*vide supra*).

Of the internal organs the liver contains the organisms in largest quantities; they may be found also in the suprarenals, and, especially at a later stage, in the kidneys. In all the organs in the human subject the spirochætes are scanty, they are often somewhat irregular and degenerate in appearance, and occur in the interior of the special cells as well as in the interstitial tissue. These facts have been explained as being the result of the formation of anti-substances, which drive them from the blood into the interstitial tissues. Their late occurrence and persistence for some time in the kidneys are comparable with what occurs in the natural infection of the rat without the occurrence of disease symptoms (*vide infra*). The spirochæte is also excreted in the urine. This does not occur in the earliest stage of the disease, but from about the tenth day onwards positive results are obtained in increasing numbers, till about the twentieth day it may be found in practically all cases. Thereafter it gradually disappears and is rarely found after the fortieth day. The best methods are to examine by dark-ground illumination the deposit thrown down from the urine by a high-speed centrifuge and at the same time to inoculate a guinea-pig intraperitoneally.

Immunity reactions.—The development of antibodies in the patient's blood has been shown to occur during the disease. These appear towards the end of the first week, and seem to be related to the disappearance of the organism from the blood; they become specially marked during the second week. Their presence can be demonstrated by injecting some of the patient's serum along with the spirochætes into a guinea-pig; lysis

occurs, corresponding to Pfeiffer's phenomenon in the case of bacteria. Antibodies can also be demonstrated by agglutination, complement fixation and adhesion reactions (p. 687). They reach a high titre and persist for years afterwards. Accordingly, they have value in diagnosis. In carrying out the agglutination test varying dilutions of serum are added to a suspension of the spirochætes (a culture in fluid medium, *e.g.* Fletcher's, without agar); drops of the mixture are examined under the microscope by dark-ground illumination.

The Adhesion Reaction in the Serological Identification of Leptospiras.—The reaction has been utilised by Davis and Brown in the identification of leptospiras. The results are analogous to those of agglutination tests with immune sera, but are more quickly obtained and more easily observed. By this reaction the leptospira isolated from yellow fever by Noguchi (p. 700) conforms to *L. icterohæmorrhagiæ*, which, however, is distinct from *L. biflexa*; *L. hebdomadis* is also distinct from the others.

By agglutination with specific antisera a number of different serological types of the leptospira, as met with in Asia, have been distinguished (Taylor and Goyle). In Europe the strains have been stated to be serologically uniform in type; but this has recently been questioned (Kien-Hun).

Experimental Inoculation.—The injection of blood or of emulsions of organs containing the spirochætes into the peritoneal cavity of a guinea-pig leads to an infection which is usually fatal in about seven to twelve days; the same holds with regard to the effect of pure cultures. The symptoms are conjunctival congestion, jaundice, tendency to hæmorrhages, albuminuria, and anæmia. There is pyrexia, which towards the end is succeeded by subnormal temperature; the jaundice becomes visible in the integuments about the third or fourth day and increases up to the time of death. Spirochætes usually appear in the peripheral blood about the fourth day of the illness and increase in numbers until the animal succumbs. *Post mortem*, the subcutaneous tissues are usually intensely jaundiced; hæmorrhages may be generalised or they may be confined to the lungs, intestinal walls, and retro-peritoneal tissue; acute parenchymatous or hæmorrhagic nephritis is present, and the spleen is large and congested. The lungs show hæmorrhages as small and large spots, being described as "like the wings of a mottled butterfly." The spirochætes are present in the blood and organs, and in the latter are chiefly interstitial in position, few being actually within cells. In this respect there is a difference from what obtains in the human disease. They

are most abundant in the liver, where they may be arranged like a "garland" round the liver cells. The adrenals and the kidneys contain considerable numbers, but they are scanty in the spleen, bone-marrow, and lymphatic glands.

When animals are inoculated with urinary sediment containing *L. icterohæmorrhagiæ*, not infrequently the organisms prove avirulent or the experimental disease is atypical: thus jaundice may be absent and the only obvious lesions are hæmorrhages, *e g.* in the lungs (*vide supra*). The loss of virulence of the organism in urine is attributed to the acid or bile present, and microscopically it may appear degenerate and atypical. Specimens of urine should be injected within an hour of being passed.

Source and Mode of Infection.—As regards the route by which the infection enters the body the Japanese workers believed that, in the human disease, infection occurs chiefly through the alimentary tract, and they were able to produce the disease in the guinea-pig by feeding with material containing the organism or by introducing some of it into the rectum. Händel, Ungermann and Jaenisch, and Buchanan, however, failed to produce infection in animals by feeding. The organisms are very susceptible to acid and are probably often killed by the gastric juice when swallowed. But an epidemic of the disease in Lisbon was traced to infected drinking water (Jorge). The Japanese observers showed that infection could take place through the apparently intact skin, and found that this occurred with comparative rapidity, as the application of an antiseptic five minutes after the infective material did not prevent infection. The occurrence of infection through the skin has been confirmed experimentally by Buchanan and others. This agrees with clinical observations on the development of the disease after immersion in polluted waters.

A highly important point with regard to the epidemiology of the disease is the common presence of the spirochæte in both house and field rats and field mice without any apparent disturbance of health. The occurrence of the *L. icterohæmorrhagiæ* in these wild rodents in practically all parts of the world has now been well established, and the proportion of infected rats may be relatively high. The organisms are practically confined to the kidneys, and we have here a resemblance to what is found in the human infection at a later stage when anti-substances are present in the blood. The spirochætes are passed in large numbers in the urine of the infected animals, and in this way contamination of the soil and various articles is brought about. The spirochætes obtained from rats are stated to vary considerably in virulence for experimental animals; this may be

due to attenuation by the acid of the urine (*vide supra*). An interesting contribution to our knowledge of the epidemiology of infectious jaundice is due to Buchanan, who has demonstrated a pathogenic strain of *Leptospira icterohæmorrhagiæ* in the roof slime of a wet coal mine in Scotland, from which an outbreak of infective jaundice had originated. The disease occurred in miners working in the particular part of the mine in which the organisms were found. Buchanan has suggested that the leptospira of infective jaundice may exist as a saprophyte under natural conditions, and from a natural source infect animals or man (*vide infra*).

It may be noted here that an infectious jaundice of dogs, known as "yellows," has been shown to be due to *L. icterohæmorrhagiæ*, and the condition in these animals corresponds to the human disease. The infection has also been observed in a fox. Schüffner in Holland has recovered from dogs a leptospira (*L. canicola*) which is serologically a distinct species. It causes uræmic symptoms, with little tendency to jaundice.

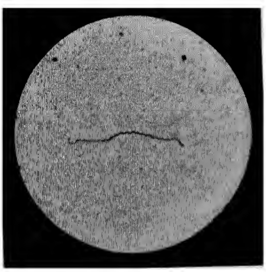


FIG. 167.—*Leptospira biflexa*, as seen in a culture obtained from water, showing elementary spirals. Kirkpatrick's stain. (p. 124). ×1200.

Therapeutic antisera to the *L. icterohæmorrhagiæ*, with properties similar to those observed in the serum during the disease (*vide supra*), have been prepared and have proved of value in the treatment of cases. Also effective protection by the use of a *vaccine* consisting of killed cultures of the organism has been recorded (Wani).

Leptospira biflexa.—Non-pathogenic spirochætes similar to *L. icterohæmorrhagiæ* have also been observed in water supplies, mines, etc., by various workers (Fig. 167). This type of organism can be cultivated like the organism of Weil's disease; it can be demonstrated by enrichment, *e.g.* in London tap water, by adding about 1 per cent. sterile fæces and keeping the mixture in a Petri plate at 25° to 30° C. for ten days to three to four weeks (Hindle). Pure cultures may be obtained by filtration through a Berkefeld filter. It has been claimed that by passage in animals it may become virulent and capable of causing in guinea-pigs the same pathological condition as *L. icterohæmorrhagiæ*. Since it is recognised that the latter may survive for a considerable time in wet soil, water, etc., it has been suggested that the saprophytic leptospira is merely a modified form of the pathogenic variety (Baermann and Zuelzer).

Leptospira Grippo-Typhosa

This organism which is microscopically identical with *L. icterohæmorrhagiæ*, has been recovered from the blood in cases of swamp fever, a condition which occurs in epidemics among workers on the land in the warm season after extensive flooding in Germany and other parts of Eastern Europe. In this disease there is a sharp febrile attack which lasts less than a week and the mortality is low; jaundice is not a feature. The organism has little virulence for guinea-pigs or mice, and the best method of demonstrating it is by means of blood culture during the first two days of the fever; it is also present in the blood before the onset of fever. It is serologically distinct from *L. icterohæmorrhagiæ* and *L. canicola*. The disease has been produced in general paralytics for therapeutic purposes by injecting a pure culture of the leptospira.

Leptospira Hebdomadis

Seven-day fever has been investigated by the Japanese workers, Ido, Ito and Wani, who established the constant presence in the disease of a leptospira which has been designated *L. hebdomadis*. The organism is morphologically similar to *L. icterohæmorrhagiæ*, and in some cases can be demonstrated by appropriate methods in the peripheral blood, though not abundant. As in infectious jaundice, the leptospira is discharged in the urine, often in large numbers, and especially in the convalescent stage. The spirochæte is pathogenic to guinea-pigs under 200 grams in weight, but larger guinea-pigs are relatively insusceptible. In experimental animals a febrile condition is produced, but icterus is only occasionally observed and, when present, is slight, and there is less tendency to hæmorrhage. The organism can be detected in the blood and *post mortem* in the liver and kidneys. The experimental disease contrasts with that due to *L. icterohæmorrhagiæ*, though there may be some similarity. Infected animals may also recover from the disease. It is claimed by the Japanese workers that this organism constitutes a species distinct from *L. icterohæmorrhagiæ*, not only on the basis of pathogenic effects but also in virtue of its serological reactions. Thus, on carrying out the Pfeiffer reaction with *L. hebdomadis* and the serum of convalescents or homologous animal antisera, a positive reaction is obtained, while *L. icterohæmorrhagiæ* reacts negatively to these sera. Conversely, the *L. hebdomadis* yields a negative

reaction with antisera to the infectious jaundice organism. It has been shown that the *L. hebdomadis* is harboured by the wild mouse—*Microtus montebelloi*—and the organisms are present in the kidneys and excreted in the urine of these animals. The source and mode of infection in the human subject would appear to be analogous to that of the *L. icterohæmorrhagiæ*.

Other Conditions in which Leptospiras have been described

Noguchi described a leptospira (*L. icteroides*) in yellow fever, which he regarded as the causal agent of this disease (p. 777). Though he claimed that this organism differed in its pathogenic and serological characters from *L. icterohæmorrhagiæ*, all recent evidence goes to show that it is indistinguishable from the latter.

A leptospira has been isolated from the blood in cases of phlebotomus fever in Malta by culture methods similar to those employed for *L. icterohæmorrhagiæ* (Whittingham). The organisms appeared in culture after five to six days' incubation at 27° C. They proved morphologically identical with *L. icterohæmorrhagiæ* but were non-pathogenic to guinea-pigs. The evidence regarding the etiology of the disease suggests that it is due to a filterable virus (p. 786).

A similar organism has also been observed in dengue (in the Eastern Mediterranean) by Couvy. The blood of patients when injected into rabbits produced pyrexia, and the spirochætes could be detected in the animals' blood. The intraperitoneal injection of crushed phlebotomus flies produced a similar infection in rabbits.

In Sumatra a febrile disease occurs which may resemble dengue or a mild form of Weil's disease. Various workers have described a leptospira (*L. pyrogenes*) in this condition and have isolated it by blood culture In some cases the spirochæte proved pathogenic to guinea-pigs, producing lesions like those of *L. icterohæmorrhagiæ*. In other cases the organism was non-pathogenic or failed to produce characteristic effects (Van de Velde, Vervoort, and others). Fletcher has also isolated leptospiras by direct blood culture from a similar condition in Malaya, and they appeared to be serologically identical with those described in Sumatra by Vervoort.

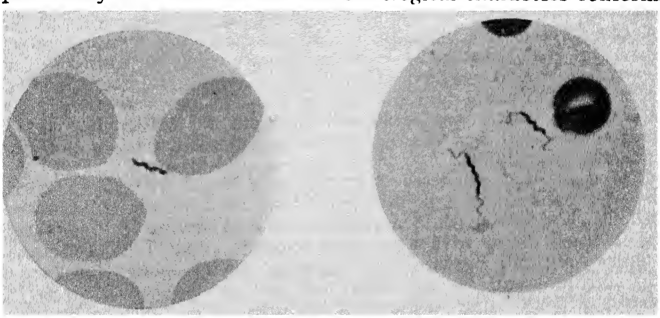
Atypical or non-icteric forms of Weil's disease simulating these conditions might possibly explain the bacteriological findings described above, and at present no definite statement can be made as to the etiological relationship of leptospiras to phlebotomus fever and dengue.

Spirillum Minus

Rat-bite fever (Sodoku), following the bite of a rat after an incubation period of ten to twenty-two days, presents a characteristic clinical syndrome: inflammation of the skin in the neighbourhood of the bite, which may have completely healed by the time of onset of the disease, paroxysms of fever of the relapsing type, swelling of lymph glands, and a patchy erythematous skin eruption. The disease has been observed in various parts of the world, but has been most studied in Japan, and in 1915 Futaki and his co-workers described a special type of spiral organism in the

skin lesion and in the lymph glands, which has now been well established to be the specific causal agent of the disease. They regarded it as a spirochæte and it was designated *Spirochæta morsus muris*; it is now called *Spirillum minus.*

The causal organism occurs in the local lesion, the related lymph glands, and in some cases in the blood. It is comparatively short, measuring 2–5 μ, and presents a few regular curves of 1 μ each (Fig. 168); it is relatively thick and easily stained, especially by means of a Romanowsky stain. By dark-ground illumination it is most readily demonstrated, *e.g.* in the blood of an infected animal, and exhibits very active motility of a "darting" type similar to that of a vibrio. Polar flagella (1–7) are present at each end; they are more frequently multiple than single.[1] The organism is practically non-flexuous and in its biological characters conforms



A B

FIG. 168.—*Spirillum minus*: A, as seen in blood film from experimentally infected guinea-pig. ×2000. B, from a natural infection in a mouse, stained to show flagella (from a preparation by Dr. J. A. W. McCluskie). ×1500.

more to a spirillum than to a spirochæte. It has been cultivated artificially by Futaki and co-workers in Shmamine's medium prepared as follows: 0·5 to 0·75 grm. sodium nucleate is dissolved in 100 c.c. horse serum; carbon dioxide is passed through the solution for four minutes, and the medium is then heated on three successive days for an hour at 60° C.; on the fourth day, it is again heated at 65° C. for thirty minutes, when it undergoes partial coagulation. Other observers have failed to obtain cultures, however, in various media, and the Japanese workers were unable to maintain their growths after subculture.

This organism has now been demonstrated in a few cases of rat-bite fever in this country. Recently Mackie investigated a case occurring in Scotland: the patient presented the typical clinical

[1] The flagella are difficult to stain; they may be demonstrated by fixing very thin films, immediately after spreading and before drying has occurred, for thirty to sixty seconds over the vapour of 1 to 2 per cent. osmic acid solution and then, when dry, staining overnight in a 1 : 10 or 1 : 15 dilution of Giemsa's solution.

condition and the characteristic spirillum was demonstrated by inoculation of guinea-pigs with an emulsion of an excised regional lymph gland (*vide infra*). It is interesting to note that a condition similar to rat-bite fever has followed the bites of cats and ferrets. Rats, mice, and guinea-pigs can be infected with the spirillum of rat-bite fever, and the organism appears in their peripheral blood. Its virulence to laboratory animals is relatively low, though guinea-pigs are fairly susceptible and, after a time, succumb to the infection. Occasionally individual guinea-pigs are resistant. Mice usually become refractory to the infection after the initial attack, and the organisms practically disappear from the blood ; although they persist in larger numbers in the peritoneal cavity (Saisawa and Taise). The spirillum occurs in a small percentage of rats and field mice under natural conditions, and the disease has been produced in the guinea-pig from the bite of an infected rat. The mechanism of infection is doubtful. It has been suggested that when the animal bites, bleeding from the mouth provides the material for inoculation. Infected animals may develop a keratitis with spirilla in the conjunctival secretion, and from this the nasal and mouth secretion becomes infected. The infection is highly amenable to treatment by salvarsan. It has been shown also that the blood of a convalescent patient possesses protective antibodies.

For diagnostic purposes, 3 to 5 c.c. of blood withdrawn during the pyrexial stage can be injected intraperitoneally into laboratory animals, but this method may fail, and it is advisable to puncture an enlarged gland with a syringe, aspirate some fluid, and inject a guinea-pig and a white rat. After a varying incubation period, the spirillum can be demonstrated, by the methods referred to above, in the peripheral blood of the infected animals. The blood and gland-juice may also be examined directly. Excision of a gland and inoculation of animals with the emulsified tissue provides the most certain method of demonstrating the spirillum. In some cases the organisms may be demonstrated directly in the local lesion, and the exudate from the lesion may also be used for animal inoculation tests.

Previously Schottmuller and also Blake had described leptothrix or streptothrix-like organisms isolated from the blood in cases of rat-bite fever presenting the characteristic clinical manifestations ; these organisms may have represented, in the particular cases, a concomitant or secondary infection. In some of these cases it is possible that *Streptobacillus moniliformis* may have been the causal organisms (p. 589).

An organism morphologically identical with *Sp. minus* occurs in the blood of mice. It is non-pathogenic to these animals and may also be non-infective to man. It does not flourish in other species (McCluskie).

CHAPTER XXIII

RICKETTSIA GROUP

Introductory.—The term *Rickettsia* has been applied to certain definite structures found to develop in the alimentary tract of some blood-sucking arthropods, *e.g.* in lice after feeding on cases of typhus fever. It has also been shown that the disease is transmitted by inoculating susceptible animals with the fæces of insects containing these " rickettsia bodies." Similar bodies occur in the cells of the lesions in man and experimental animals. Several other diseases have likewise been found to be transmitted by arthropods harbouring such bodies, namely trench fever by lice and Rocky Mountain spotted fever by ticks, also the South African disease of cattle known as " heart-water." The rickettsia bodies are very minute diplococcal or rod-shaped structures, measuring as a rule less than 1 μ, although longer forms occur and considerable pleomorphism is shown. They are Gram-negative, but they have only a weak affinity for the ordinary bacterial stains, and in order to demonstrate them Giemsa's solution is most suitable; with this they stain of a reddish or a purple colour. The finding of the rickettsia bodies has led to much investigation regarding their occurrence in various insects and their significance. On the one hand, the specific etiological relationship of the rickettsia bodies to the respective diseases appears, in the case of lice, from the fact that insects fed upon healthy persons do not develop them and that their appearance in the gut coincides with the acquisition of infectivity by the lice. On the other hand, similar bodies have been found in various blood-sucking arthopods apart from the occurrence of disease in their mammalian hosts. Thus Nöller found that *Melophagus ovinus* (the sheep " ked ") habitually harbours rickettsiæ, and the bed-bug (*Cimex lectularius*) is constantly infected with such bodies (Arkwright, Atkin and Bacot). Hertig and Wolbach and others have also demonstrated their presence in a number of other arthopods. As regards the nature of the rickettsia bodies there is great uncertainty, although it is clear that they are definite structures distinguishable from

mitochondria, etc.; that they are living objects capable of multiplication seems evident. In the case of typhus fever they appear in large numbers in the intestinal contents of the lice after some days following the ingestion of infected blood, and in this case also they are seen to pack the epithelial cells of the gut and to cause disturbances in function which lead to the death of the insect. Other rickettsiæ again are harmless to the arthropod hosts in whose tissues they appear to flourish in permanent symbiosis, being transmitted in the eggs from one generation to another. In general, attempts have failed to obtain cultures of pathogenic rickettsia bodies on the usual artificial media, although they have been found to multiply in tissue cultures and in media containing minced fresh tissues. *Rickettsia melophagi* grows on blood agar and in its behaviour in culture resembles the ordinary bacteria.

Rickettsia prowazeki (Rickettsia of Louse-borne Typhus Fever)

Experimental Transmission of Typhus Fever.—In the following description, the form of typhus fever will be referred to which has long been known to cause serious epidemics in Europe and Asia. Since it has been learned that similar diseases exist which can be more or less clearly distinguished clinically and otherwise, this original type has been variously named " epidemic," " Old World," " classical," " louse-borne," " human." The essential feature of this infection, however, is its transmission from man to man through the agency of lice, and the virus has maintained high virulence, presumably as a result of constant passage through one susceptible species of mammalian host. An attenuated form of the disease may exist, however, *e.g.* the condition known as Brill's disease in New York and other towns of the north-east coast of the United States. The first definite results regarding the etiology of typhus fever were obtained by Nicolle in 1909. This observer found that the blood of cases of typhus fever during the febrile period, as well as immediately before and afterwards, was infective for chimpanzees and some species of lower monkeys, in the latter especially when introduced intraperitoneally. A febrile illness which was practically identical with the disease in man (including occasionally skin eruption and sometimes fatal) was produced, and the blood of infected animals was again infective toward fresh individuals. A large number of such passages were successful. Of other animals the most susceptible to the

infection is the guinea-pig, in which, following intraperitoneal inoculation with several c.c. of infective blood, there occurs an illness after an incubation period, which is seven to twelve days as a rule, characterised by fever and loss of weight; the illness lasts for four to eleven days and is only exceptionally fatal. The infective agent is present in the guinea-pig during the febrile period in the blood and solid organs, especially the brain. In this animal likewise it can be maintained by passage and does not undergo any change in virulence. In the guinea-pig, as in the human subject, the characteristic lesion is a proliferation of the endothelial cells of the small blood vessels, which form masses in and around the lumen and lead to thrombosis and occlusion. The cellular masses are marked in the brain, but are also present in other organs and in the skin. In the male guinea-pig, after intraperitoneal inoculation an inflammatory exudate may occur into the scrotal sac; but this is much more marked with typhus of American and Mexican origin than with European typhus (see p. 710).

Rats show no symptoms after inoculation with typhus agent, but their blood becomes infective, as can be demonstrated by inoculating guinea-pigs. Kusama and Ségal have found that the agent is particularly associated with the blood platelets, since a suspension of these elements separated from the other constituents of the blood by centrifuging is specially infective, whereas the blood plasma and also leucocytes from peritoneal exudate do not harbour the infective agent.

Nicolle found that the infective agent was destroyed by a short exposure at from 50° to 55° C. It is probably not filterable.

The Vector of Infection.—A most important fact established by Nicolle was that infection takes place through the *Pediculus humanus* (*corporis* variety; probably also *capitis*). Monkeys and guinea-pigs can both be infected by lice previously fed on a human case. There is evidence that the causal organism undergoes development or multiplication in the insect host, as the louse becomes infective after the seventh day following the infected feed. It is present in the fæces of the lice which constitute the source of infection for man under natural conditions. During the late war there were serious outbreaks of typhus fever in Serbia, Bulgaria, and Poland, and measures founded on the view that the body louse is essential to the spread of the epidemic were always successful in controlling its spread. Accordingly, the evidence is practically conclusive that typhus fever is spread from man to man by the louse. It

is possible that the causal agent may be transmitted through the eggs of the insect to a second generation of lice, but this appears to be very unusual.

The Infective Agent.—There is now much evidence regarding the causal relationship to typhus of *Rickettsia prowazeki*, the name given by da Rocha-Lima to certain small bodies found by himself and Prowazek in the intestine of lice taken from typhus fever patients. These are probably identical with structures described earlier by Ricketts and Wilder (who stated also that they were present in large numbers in the blood of typhus fever patients, but this has not been confirmed), Sargent, Foley and Vialette, and others. They are non-motile bodies which are most satisfactorily demonstrated by staining with Giemsa's solution, when they appear characteristically as minute cocci, diplococcus-like bodies, or rods measuring 0·25 × 0·3–1·0 μ, usually of a reddish or lilac tint with a bluish intermediate substance; a pale capsule has also been described round the ends (Fig. 169). But there is considerable pleomorphism, and thicker forms, threads and chains up to 4–10 μ or even 20–50 μ are also met with, the larger and thicker forms staining purple. They are Gram-negative. The best method of studying these organisms *in vivo* consists in introducing the virus into the anterior chamber of a rabbit's or guinea-pig's eye. An acute inflammatory reaction develops and the rickettsia bodies are then found in large numbers in the endothelial cells of Descemet's membrane (Nagayo and others). Growths on the ordinary culture media have not been obtained; but pure cultures have been got by Goodpasture's method on the chorio-allantoic membrane of the chicken embryo.

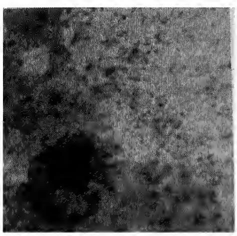


FIG. 169.—*Rickettsia prowazeki* from teased gut of an infected louse. An intestinal epithelial cell is seen containing numerous Rickettsia bodies. Giemsa's stain. × 1500. (From a specimen lent by Sir J. A. Arkwright, F.R.S.)

These bodies appear after an interval of about a week or longer in a proportion of lice which have been fed upon typhus patients and are kept at a suitable temperature (about 30° C.), and they are the only organisms regularly found under such circumstances. The rickettsia bodies parasitise the cells of the alimentary tract of the lice from the mid-gut downward. The infected cells become distended with the bodies, so that the

nutrition of the infected insects is interfered with and they die. The rickettsia bodies are also found in enormous numbers in the fæces of infected lice. (Similar bodies have been met with in lice fed upon cases of trench fever, but the latter show no tendency to invade the epithelial cells of the gut.) Lice fed upon healthy persons in whom infection with typhus fever can be excluded appear never to harbour such organisms. A similar organism described by Weigl under the name of *R. rocha-limæ* as occurring in human blood, apart from obvious typhus and non-virulent for man and very feebly virulent for guinea-pigs, may be a modified form of *R. prowazeki*.

The transmission of typhus infection to guinea-pigs is effected by inoculation with the gut contents of lice harbouring the rickettsia bodies, and in the absence of these bodies the guinea-pigs do not become infected. Also blood platelets of infected guinea-pigs when injected per anum into lice cause the appearance in the latter of *R. prowazeki*, as shown by Bacot and Ségal. The Red Cross Typhus Commission to Poland has demonstrated by special methods the rickettsia bodies in the endothelial cells of the vascular lesions of the skin, brain, and other organs from human cases, and also found them, though less frequently, in the tissues of infected guinea-pigs.[1] In tissue cultures derived from the brain of infected guinea-pigs. Wolbach and Schlesinger have found that *R. prowazeki* multiplies in the endothelial cells, and material from a subculture twenty-eight days after removal from the animal was shown to be infective when inoculated into a fresh guinea-pig. Otto and Dietrich found specific agglutinins for *R. prowazeki* in the serum of typhus convalescents.

In view of these facts the etiological relationship of the rickettsia bodies to typhus fever appears to be established. As regards the nature of the virus, whether bacterial or protozoal, nothing definite is known. The excreta of infected lice remain virulent as a dry powder at room temperature for at least eleven days; the high infectivity of such material would explain the

[1] Tissue, *e.g.* brain, which has been fixed in formalin is cut into slices 2–3 mm. thick, washed in running water for twenty-four hours, then treated for thirty-six hours with Regaud's fluid (potassium bichromate 2·5 grams, sodium sulphate 1·0 gram, distilled water 100 c.c., to which is added before use 20 c.c. formalin); thereafter the tissue is washed for twenty-four hours, dehydrated, etc., and embedded in paraffin. Sections are stained with Giemsa's solution overnight (p. 131). Differentiation is best effected by exposure of the stained sections to strong sunlight; the rickettsia bodies then appear of a deep bluish purple tint, while the cytoplasm of endothelial cells is faint blue. Castaneda's method may also be used for demonstrating the bodies (p. 725).

frequency with which this disease has been contracted by investigators in the laboratory (Arkwright and Bacot). It seems probable also that the entrance of infected louse excreta into minute punctures of the skin, *e.g.* the bites of the insects and accidental scratches, constitutes the chief mode of infection in the human subject under natural conditions.

Immunity Phenomena.—Nicolle observed that, as in the case of man, when an experimental animal has passed through an attack of the disease it becomes immune to re-inoculation. By taking advantage of this fact it has been shown in cross-inoculation experiments that typhus fever as met with in different parts of the world is immunologically the same. It had been known that children under ten years are, apparently, less susceptible to typhus than older individuals, and Nicolle made the interesting observation that when a family is attacked, young children, while apparently well, may really suffer from a slight rise of temperature. This condition is probably an abortive attack of the fever, as the blood in such cases is infective for animals. These abortive cases may play a part in the dissemination of the disease. Nicolle's results have been confirmed in America by Anderson and Goldberger and by Ricketts and Wilder.

The serum of both men and animals during convalescence confers passive immunity, and may have a slight curative action, but this rapidly disappears. Nicolle and Conseil, by injecting into asses intravenously or intracerebrally emulsions of the brains of guinea-pigs containing living virus, have produced an antiserum which has protective effects in guinea-pigs. Active immunisation has been effected in the human subject by injecting several small doses (1 c.c.) of serum derived from cases of typhus fever or from guinea-pigs at the height of the fever, but it is not certain whether this procedure is safe as a general measure. Weigl has used as a vaccine for the human subject the intestinal contents of lice infected with typhus rickettsia, which are ground up in a weak solution of phenol. Successful attempts to produce effective antisera by means of rickettsia bodies killed with formalin have been recorded by Zinsser and Castaneda. They injected large amounts of rickettsia bodies (derived from the tunica vaginalis of guinea-pigs infected with the Mexican form of the disease). The resulting anti-serum, when injected into guinea-pigs several days after inoculation with European typhus virus, prevented development of the disease.

Other Organisms Isolated from Typhus Fever Patients.—Various bacteria have been obtained from the blood or tissues

in typhus fever cases, most of which are clearly accidental concomitants. Plotz isolated from the blood a Gram-positive anaerobic bacillus which Olitzky and others have also cultivated from infected lice, but it is not clear that this organism bears any relationship to the disease. Bacilli of the proteus group have been cultivated from the urine and blood of typhus fever cases and also *post mortem* from the organs, which, while certainly not the causal organisms, are important because of their being agglutinated by the blood serum of patients (reaction of Weil and Felix). The tendency for the blood serum in typhus fever to contain heterologous agglutinins was originally observed by Wilson, who found a positive Widal reaction for *B. typhosus* in such cases.

Weil-Felix Reaction.—The organism isolated by Weil and Felix (designated *Proteus X–19*) is so constantly agglutinated in high dilutions by the serum from typhus fever patients, while it is not affected by serum from other conditions, that the reaction possesses great value in diagnosis. The agglutinins for this organism appear in the blood on the fourth or fifth day of the disease and reach their height in the second week. They may still be present many months after convalescence ; but the reaction may become negative in from six weeks to three months after the onset of the illness. Rabbits infected with typhus agent develop agglutinins for *B. proteus X–19*. but guinea-pigs do not.

The test should be carried out by the naked-eye method (p 139) with living suspensions of agar-slope cultures, the mixtures of serum and organisms being incubated at 37° C. for two hours. Two-thirds of the cases yield a positive reaction in a dilution of 1 : 800 of the serum or greater, and less than 4 per cent. fail to cause agglutination with a dilution of 1 : 100 (Red Cross Typhus Commission). On the other hand, non-typhus sera do not agglutinate this organism in a dilution greater than 1 : 50. Thus agglutination with a 1 : 100 dilution of serum may be taken as a positive reaction Felix recommends the use of a culture of the O variant for the performance of the test, since the agglutinins in typhus fever are of the O type : agglutination occurs more slowly with such organisms ; also for a positive result the titre must be 200 or higher In order to prevent reversion of *B. proteus O–X19* to the H form it should be grown on dry agar, and, if necessary, typical non-spreading colonies should be selected after plating. Change of the O form to the R form must also be guarded against by testing suspensions in 3·4 and 6·8 per cent. NaCl solution, which will cause the R form to sediment. In order to preserve the agglutinability of suspensions of the organisms Sachs has found heating at 80° C. for an hour effective ; such heated organisms agglutinate in larger flocculi than the living bacilli do, although clumping occurs more slowly than with the latter. On the other hand, heating

at 56° C. destroys the agglutinability, and unheated suspensions gradually become inagglutinable on keeping. Another method of preservation consists in the addition to the suspension of 50 per cent. alcohol.

Rickettsia Infections Responsible for Varieties of Typhus

Neill (1917) and Mooser showed that male guinea-pigs inoculated intraperitoneally with blood from cases of typhus fever occurring in Texas, regularly developed marked scrotal lesions, a condition which was absent in animals infected with the European typhus virus. Further, it has been discovered that there exist in various parts of the world diseases more or less closely resembling typhus, many of which are relatively mild and do not occur in epidemics and which are not spread by lice but by other arthropod vectors. Cross-immunity tests have shown that some of these diseases correspond immunologically with classical typhus, while others are distinct. Again, while in certain of them the patient's serum agglutinates powerfully, the *O–X19* strain of *B. proteus*, in others different strains are agglutinated or there may be only a weak effect on any of the strains so far tested. These facts are interpreted by Felix as evidence that the different strains of *B. proteus* are genetically related to the different viruses. Various names have been given to these diseases; the type of typhus acquired from rodents, especially rats, of which fleas are the vector, has been called "murine typhus." This occurs both in the eastern and western hemispheres. Also, there are the mite-borne tsutsugamushi disease of Japan, and tick-borne infections, such as Rocky Mountain Spotted Fever, which is often very fatal, and "Fièvre boutonneuse" of Tunis, which is a mild disease.

Murine typhus is known in Mexico as "tabardillo." The agent has been found in the rat flea, *Xenopsylla cheopis* (Dyer and others) and in the tissues (brain) of rats (Mooser, Castaneda, and Zinsser). This agent, when injected intraperitoneally into male guinea-pigs, causes after four to six days a very marked inflammatory swelling of the scrotum. The exudate in the tunica vaginalis shows numerous rickettsia bodies (*R. mooseri*) in the endothelial cells, which are indistinguishable from *R. prowazeki*. Strong agglutinins develop in patients' serum for *B. proteus O–X19* and there is cross-immunity between this infection and that of classical typhus. On reducing the resistance of guinea-pigs by exposure to X-rays or other means, very large accumulations of rickettsia bodies can be got and by

using these killed with formalin as a vaccine for injection of horses, antisera have been obtained which have a curative action in infected guinea-pigs. Such exudate also is very suitable for obtaining growths of the rickettsia bodies in tissue cultures. Rats infected with this virus develop a definite febrile illness and may die. The "Fièvre nautique" of Toulon, Manchurian typhus, and the urban type of typhus in the Malay States all appear to be due to a similar virus. Possibly the "mouse fever" of Australia is also of this type. The infection apparently exists widely in nature among rats and perhaps other rodents, and its transfer to the human subject by fleas is an accidental occurrence. Its relatively mild character in man would thus be due to its lack of accommodation to the human host. It has not proved possible under experimental conditions to transform the murine agent into the louse-borne variety or *vice versa*, so far as their behaviour in animals is concerned.

Tsutsugamushi disease in Japan and the rural (scrub) type of typhus in the Malay States are closely related, if not identical. The patients' blood agglutinates strongly the Kingsbury strain of *B. proteus* (*O–XK*), but not the *O–X19*. Rats or other rodents act as the reservoir of the virus. The vector in the first case is a mite (*Trombicula akamushi*) and probably also in the second. It was in the case of tsutsugamushi disease that Nagayo and others first showed that the infection may be passed in series through rabbits by inoculation into the anterior chamber of the eye; an acute local inflammatory reaction develops with the appearance of rickettsia bodies (*R. orientalis*) in the endothelial cells of Descemet's membrane. Cross immunity tests are positive (Lewthwaite and Savoor).

Rocky Mountain Fever.—This typhus-like disease has been the subject of much investigation in America. Its severity varies greatly; in certain districts cases are few but the fatality is high, while elsewhere the disease may be prevalent but mild. The essential pathological anatomy appears to be an inflammatory reaction of the adventitia of the vessels of the subcutaneous tissue and of the genitalia, with degenerative changes in the media, and a perivascular accumulation of large mononuclear cells. There is also thrombosis in the vessels. The disease is transmitted by ticks; *Dermacentor venustus* (*D. andersoni*), which infests a variety of mammalian hosts is the chief vector to man. Monkeys, rabbits, and guinea-pigs can be infected with the blood, and also by ticks. The natural animal reservoir has not been discovered, however. The virus is transmitted hereditarily in the eggs of the ticks. In the

guinea-pig the illness is much more severe and fatal than in typhus infection. There is fever with. in the male, swelling and hæmorrhage in the scrotum, swelling and rash in the ears, swelling and sometimes necrosis of the paws, etc. In the blood vessels in human cases and in infected guinea-pigs, especially within the large mononuclear cells and smooth muscle cells, and also in the stomach of the tick, Wolbach found bodies closely resembling *R. prowazeki*, which he named *Dermacentroxenus rickettsi* now known as *R. rickettsi*. These bodies multiply within the endothelial cells in tissue cultures. None of the strains of *B. proteus* hitherto used in the diagnosis of typhus fever is strongly agglutinated by the serum of Rocky Mountain fever cases. Also there is no cross-immunity between this infection and classical typhus. The serum of animals which had recovered from the infection was found by Ricketts and Gomez to possess protective power, and Noguchi has shown that an antiserum may be obtained from rabbits as a result of repeated intravenous injections of blood containing the agent. This antiserum when injected during the incubation period is effective in preventing the outbreak of the disease in guinea-pigs inoculated with many times the lethal dose. Active immunity can be developed in these animals by inoculation with fresh mixtures of living virus and antiserum. Effective protection against the disease has been obtained in man by injecting several doses of a carbolised emulsion made by grinding up highly infected ticks. Immunisation must be repeated each year.

São Paulo Typhus.—Toward the end of 1929 this apparently new disease broke out in the region of Brazil, whose name it bears. It does not spread from man to man, but presumably is derived from wild animals. The clinical features closely resemble those of Rocky Mountain fever, but differ in respect that a local lesion often develops at the site of the bite of the vector tick, also the patient's serum agglutinates strongly *B. proteus O–X19* (Felix). Experimentally various ticks may be responsible for its transmission. The causal rickettsia, *R. braziliensis*, has been cultivated in the allantoic membrane of eggs, and also multiplies on the cornea in guinea-pigs after inoculation of the virus into the anterior chamber of the eye.

Fièvre boutonneuse, which occurs on the shores of the Mediterranean, is spread by a tick, *Rhipicephalus sanguineus*, from dogs, which are frequently infected. The name of the disease indicates the local nodule which develops at the site of the infective tick bite; this is usually on the leg. The serum of

patients agglutinates *B. proteus O–X19* only weakly and does not affect *O–XK*. Similar tick-borne typhus fevers occur in India (Megaw) and in South Africa; but the vector is not known in all.

Trench Fever

Trench fever (also known as Wolhynian fever or five-day fever) was recognised as a distinct disease only during the late war. Though not a fatal malady, it was responsible, owing to its wide prevalence, for a serious amount of temporary disablement in the armies, while the after-effects, which occurred in a proportion of cases, were the cause of much chronic ill-health. Recovery from the disease does not lead to any marked degree of immunity.

McNee and Renshaw showed that the disease could be transmitted to a healthy individual by the intramuscular or intravenous injection of the blood of a patient in the acute stage. In these experiments the period of incubation varied from six to twenty-two days. Subsequently extensive investigations on this disease were carried out by a British War Office Committee working in this country and an American Research Committee in France. It was shown that trench fever is transmitted by means of lice, both the body louse and the head louse being vectors. The British Committee made numerous attempts to transmit the disease by the bites of lice which had previously fed on trench fever patients, but without success (the American Committee, however, observed infections produced by bites). If the excreta of such lice were collected and dried, and were used to inoculate a scarified area of the skin of healthy men, trench fever resulted in a considerable number of cases, the period of incubation being on an average about eight days. After lice were allowed to feed on a trench fever patient, a period of from five to nine days elapsed before their fæces became infective, this period suggesting a cycle of development in the louse, or indicating the time during which the organisms multiply sufficiently to produce infection. The lice remain infective for a period of at least twenty-three days after being infected, and are probably infective throughout their life It was also found that the bodies of infected lice when crushed on the broken skin were capable of giving rise to trench fever. Even as late as fifteen months after the onset of the disease the blood during a slight febrile attack may contain the organism of the disease, as was shown by its capacity of infecting lice—a fact in accordance with the protracted character of the disease in some cases and the recrudescence of typical symptoms. It was found impossible to

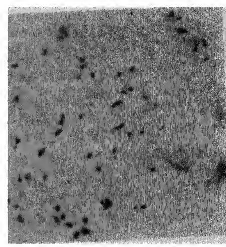


FIG. 170.[1] – *Rickettsia quintana* in intestinal contents of an infected louse. Giemsa's stain. ×2000.

[1] We are indebted to Sir J. A. Arkwright, F.R.S., for the preparations from which Fig. 170 was made.

produce infection by the excreta of healthy lice—that is, the virus is not normally resident in the insect. Further, the infective agent is not transmitted from infected lice to their offspring The comparative regularity with which the disease may be produced in men of various ages by infected lice shows that the proportion of naturally immune individuals is very small.

The evidence is in favour of the infective agent existing in the blood as an extra-corpuscular parasite. Although it may be present in the urine of trench fever patients, and sometimes in the sputum mixed with saliva, there is no evidence that the disease is spread otherwise than by means of lice. The agent has not been found in the fæces. The American Committee found that on rubbing up the dried urinary sediment in saline and then passing the fluid through a Chamberland L filter, they were able to set up trench fever by injection of the filtrate. The organism may thus be, at least in one stage, a filter-passer. It is relatively resistant; it is not killed by drying, and dried excreta have been found virulent after keeping for four months; also it resists exposure to sunlight for some time. It survives an exposure to dry heat at 80° C. for twenty minutes, but when moist is killed by a temperature of 60° C. for a like period.

Töpfer described the occurrence of rickettsia bodies in lice which had fed on trench fever patients, and this has been confirmed by Arkwright, Bacot and Duncan, and others (Fig. **170**). This organism *R. quintana*, in its microscopic appearance and staining reactions closely resembles the typhus fever rickettsia, though less pleomorphous; unlike the latter, it does not parasitise the epithelial cells of the gut in lice, but remains confined to the lumen, and the insects are not harmed by its presence. The evidence as to its etiological relationship to trench fever rests on the facts that the rickettsia bodies appear in lice five to twelve days after feeding on a trench fever patient, and that lice excreta which contain the bodies transmit the disease, whereas lice in which the possibility of having fed upon trench fever patients can be excluded do not show rickettsia and are not infective. No animal has been found susceptible to infection with trench fever virus either from blood or lice.

CHAPTER XXIV

FILTERABLE VIRUSES

Introduction

While a large number of infective diseases have been proved to be due to specific organisms which can be demonstrated microscopically and cultivated artificially outside the body, there remain certain prevalent diseases, undoubtedly infective in nature, in which the causal agent is still undefined in the biological sense. For many years now a category of viruses responsible for various infective diseases has been recognised, which are regarded as analogous to other microbes, but are sufficiently small to pass through the pores of an earthenware or porcelain filter, *i.e.* are "*filterable,*" and may be invisible by the ordinary microscopic methods, *i.e.* "*ultramicroscopic.*" These viruses have been regarded as living organisms in virtue of their power of apparent multiplication in the tissues, demonstrable by the natural and experimental propagation of the diseases due to them.

The earliest observation on this subject was made in 1892 by Iwanowsky and independently by Beijerinck, who demonstrated a filterable virus as the causal agent of the mosaic disease of the tobacco plant. Löffler and Frosch in 1898 found that in foot-and-mouth disease of cattle, when the contents of the vesicles characteristic of the condition were diluted and passed through a Berkefeld filter, the filtrate which was free from any micro-organisms recognisable by the microscope or by culture, was still infective like the unfiltered material. In short, the vesicle fluid appeared to contain a specific virus which was ultramicroscopic and filterable. Since then many infectious diseases of man, animals and plants have been shown to be due to such viruses, and their study has assumed great importance.

It must be remembered, however, that the ability of an organism to pass through a filter depends not only on its size and on the fineness of the particular filter used, but also on its

plasticity and flexibility and on the pressure at which filtration is carried out. Earthenware and porcelain filters are made of various grades of porosity, and while a filter of a certain grade may allow the passage of a particular virus, a finer filter may arrest it. Another property of the filter which may play an important part is its capacity for adsorbing substances on its surface. This action, which is of a physico-chemical character, depends on the nature of the material constituting the filter ; the pH and the electrical charge of the filter and the substance which is being filtered require also to be considered. In virtue of adsorption, substances which are so finely dispersed as practically to be in solution, *e.g.* ferments or dyes, may be retained by a filter. Thus the retention of a supposed virus by a filter does not necessarily mean that it is too large to pass through the pores. It has been estimated that the pores of the Berkefeld filters are larger than some of the bacteria which are retained by such filters. Thus, filtration does not imply merely the passage of a particle through a pore or channel of greater dimensions. In recent times organic membranes such as collodion have been employed to test filterability. This work has been developed by Elford and a method has been evolved for obtaining membranes of uniformly graded porosity —*gradocol* membranes. The porosity of these membranes ranges from 3 μ to 10 mμ (0·01 μ). They are prepared by evaporating a solution of collodion in acetone to which has been added a mixture of ethyl alcohol and ether along with varying quantities of amyl alcohol. The degree of porosity is determined by the composition of the collodion solution and the conditions of evaporation. By means of such membranes Elford and his co-workers have computed the sizes of the particles representing various filterable viruses : these range from 8–12 mμ (poliomyelitis virus) to 0·125–0·175 μ (vaccinia virus).

The filterable viruses have also been described as ultra-microscopic, though in certain virus diseases, *e.g.* vaccinia, very minute coccoid bodies—“ elementary bodies ”—can be recognised by suitable microscopic methods and these are considered to be the actual virus. It has been generally assumed that by ordinary microscopic methods micro-organisms or particles of less diameter than 0·2 μ are invisible, this being about the limit of the resolving power of the microscope. Coles, however, has pointed out that with white light as the illuminant and an objective of N.A. 1·4 a dark isolated particle just over 0·074 μ in diameter is visible, and with green light an even smaller particle (0·0673 μ diameter) can be seen. It

has been estimated that the Berkefeld V filter (one of the most porous varieties) will allow the passage of particles measuring approximately 0·2 μ so that theoretically an organism might pass through certain grades of filters and yet be demonstrable microscopically; and this is the case with the organism of bovine pleuro-pneumonia (*vide* p. 484) which at one time in virtue of its filterability was classified with the viruses. Laidlaw and Elford have recently described a group of filterable saprophytic organisms which can be recognised as visible forms, though exceedingly minute, and can be cultivated like the bacteria. In the microscopic study of virus particles, Barnard has utilised photography by ultra-violet light, which is of shorter wave-length than that of the visible rays, and thus has obtained greater microscopic resolution. (For this purpose quartz lenses are required, as glass absorbs the ultra-violet rays.) By this method he has been able to demonstrate photographically virus particles of 0·075 μ in diameter. Dark ground illumination, which renders visible very minute bodies not readily demonstrable with the ordinary microscope, has not contributed much to our knowledge of the ultra-microscopic viruses, owing to the fact that an organism of such minute dimensions, unless actively motile, cannot be distinguished from the numerous particles seen by this method in all organic material. It should be noted that certain spirochætes *e.g.* the leptospiras, are capable, in virtue of their slender and flexible structure, of passing through some filters. The question has arisen, however, in regard to the filterability of spirochætes, whether the spirochæte itself or some supposed "granular" phase constitutes the filter-passer. It has also been claimed that certain of the ordinary bacteria, *e.g.* the tubercle bacillus, develop a granular filterable form, but this requires confirmation.

It has been shown that viruses are subject to centrifugal force, like the bacteria, and a method has been developed for estimating the size of virus particles by subjecting them to intense centrifugal action under conditions in which Stokes's law is applicable (Schlesinger and Elford). In this way estimates have been made of the sizes of vaccinia and certain other viruses, which are in close agreement with those determined by filtration through collodion membranes.

The biological relationships of the filterable viruses have been the subject of much speculation and the question arises whether they are living organisms like the bacteria. It would be unreasonable to suppose that the range in size of micro organisms should be limited to that of structures, like the

ordinary bacteria, which are within the limits of resolution by the microscope. As mentioned above, certain viruses when stained by suitable methods are seen as minute coccoid bodies measuring about 0·2 μ in diameter. This is exemplified in the case of the Paschen bodies of vaccinia and the elementary bodies of psittacosis, though possibly their size may be an artefact due to deposition of the stain on their surface. In this connection it is noteworthy that the virus of vaccinia has been computed by filtration through collodion membranes to have a diameter of 0·125–0·175 μ. On the other hand certain viruses have been estimated to be not much larger than the molecule of a complex protein, and it is difficult to conceive a living organism of such minute dimensions. Between these extremes, however, there is an almost continuous gradation, and it is impossible in the present state of knowledge to set any definite lower limit in size to organismal forms.

An essential property of the filterable viruses is their capacity to propagate under appropriate conditions, *e.g.* in the tissues. In this respect they show a close analogy with the bacteria and other pathogenic microbes. They also invade the body, produce infection and exert pathogenic effects like the recognised bacteria. They are antigenic and immunity may follow inoculation with them. Different immunological types may also be recognised, like those of many bacterial species, *e.g.* in the foot-and-mouth disease virus. In general the viruses are susceptible to the same physical and chemical agents as the bacteria, though there are important exceptions to this statement (*vide infra*). They are also similarly subject to adaptation, *e.g.* exaltation and attenuation of virulence. Thus the closest analogy exists between these pathogenic agents and the bacteria, and the view may now be strongly upheld that they are microbic in nature though not necessarily belonging to a single group with uniform characters.

As will be shown later, the viruses multiply only in association with living cells, and it has been suggested that a virus is an inanimate substance which when acting on the cell in some way induces it to produce more of the particular substance so that propagation is simulated. Studies of bacteriophage have led some workers to believe that in this way an enzyme-like principle may appear to multiply (*vide* p. 45). On the other hand, there is no evidence that any of the recognised enzymes ever simulate propagation. Vinson and his co-workers have isolated a crystalline protein substance from the mosaic disease of the tobacco plant (long recognised as due to a virus) and claim that this substance is " infective." Attempts have also been made to ascertain whether viruses " re-

spire " like living organisms, but the evidence at present indicates a lack of respiratory functions. Thus the controversy whether these viruses are animate or inanimate remains unsettled.

While the terms "filterable" and "ultramicroscopic" have been commonly used, it is apparent that they are not strictly applicable in the designation of these viruses as a whole, and there is a growing tendency to refer to this group of undefined infective agents as "viruses."

Attempts have been made to cultivate various viruses artificially and to reproduce their pathogenic effects by means of such cultures. While they have not been cultivated in ordinary artificial media, many of them multiply *in vitro* when in association with living tissue cells, *e.g.* in tissue cultures or along with surviving tissue freshly removed from the animal body.

A remarkable feature of most filterable viruses is their resistance to glycerol, especially at low temperatures. Thus, while spore-free bacteria generally are killed by 50 per cent. glycerol, the viruses of small-pox, rabies, poliomyelitis, herpes, etc. remain unaltered for long periods in this fluid at 4° C. This property has been utilised practically in the preservation of the vaccine virus in calf lymph for small-pox vaccination (*vide* p. 735). Thorough and rapid drying *in vacuo* at a low temperature and storage in the cold appears to be the best method of preserving viruses. They are relatively susceptible, however, to atmospheric oxygen and to heat and chemicals, though, as in the case of other organisms, considerable variations are met with.

In the majority of virus infections special intracellular structures have been found—"inclusion bodies." These are in some diseases restricted to a particular tissue, but in others they are present in the cells of various tissues. In certain diseases the bodies occur in the cytoplasm, in others they are intranuclear, and they may be met with in both situations. They vary greatly in size from about 0·2 μ upward, and in the cytoplasm of a single cell there may be forms measuring 1 to 20 μ or more. For their demonstration a useful method is to fix smears while still wet in a suitable fixative (*vide infra*) and stain with Giemsa's solution or other eosin-methylene-blue combination, or Mann's methyl-blue-eosin stain; these stains are also used for sections. The cytoplasmic inclusion bodies tend to stain with acid dyes, but often they contain also basophile constituents. The intranuclear bodies are eosinophile. Various views have been held as to the nature of these inclusion bodies; it is probable that they represent the virus particles or aggre-

gates of these surrounded by cellular products, either reactive or degenerative, and derived from both nucleus and cytoplasm. In accordance with the conception that inclusion bodies represent parasites "cloaked" with cellular material they were at one time called "chlamydozoa." In the case of the Bollinger bodies in fowlpox, the disease has been reproduced by inoculation with one such body, whereas the fluid in which it had been washed proved non-infective (Woodruff and Goodpasture). In some cases by suitable methods the bodies can be shown to be composed of minute granules, "elementary corpuscles or bodies," and these correspond to the minute bodies which may be observed in lesions as free structures, *e.g.* in the pox diseases. In certain cases, *e.g.* the Negri and Guarnieri bodies of rabies and vaccinia respectively, the cell inclusions are specific in the sense that their morphology and distribution are highly characteristic for the particular disease. But, of course, the virus may be present in material in which inclusion bodies cannot be demonstrated. Cell inclusions and elementary bodies have been seen to develop *in vitro* in tissue cultures containing a virus. These facts suggest that the filterable viruses are intracellular parasites, and in this respect they contrast with most of the ordinary bacteria. It may be that they represent the most extreme degree of parasitism in nature.

Immunity to virus diseases exhibits the same wide range of phenomena as is manifested in the ordinary bacterial infections. Recovery from some of them is followed by marked specific resistance which is of long duration, *e.g.* small-pox, measles, poliomyelitis, etc., and this result may also follow inoculation with the virus in an attenuated form, *e.g.* the rabies virus; whereas after others susceptibility to re-infection soon returns, *e.g.* herpes, influenza, etc. Certain of the viruses when in the "dead," *i.e.* non-infective, state also act as effective vaccines. The serum of recovered or immunised animals may deprive the virus of infectivity, as proved by failure of a susceptible animal to develop lesions on inoculation with a mixture of the two; sometimes fixation of the antibody by the virus *in vitro* can be shown to occur. Passive immunity may be conferred by injections of the immune serum. Flocculation and complement-fixation reactions resulting from the interaction of virus and antibody *in vitro* have also been demonstrated, although the results have frequently been negative. In investigating the identity of viruses from different sources, extensive use has been made of the specific anti-infective action of immune sera or their flocculating property; viruses whose

infectivity is neutralised by the same antiserum or which are flocculated by it in similar degree, are considered to be homologous. Ledingham has succeeded in obtaining homogeneous suspensions of the elementary bodies from the lesions of vaccinia and fowlpox, and has shown that they are agglutinated specifically by the sera of animals which have recovered from the respective infections or have been immunised with the corresponding viruses ; normal sera lack this agglutinating action. Such serological evidence supports strongly the view that these elementary bodies constitute the actual infective agent.

The diseases of man and animals due to the filterable viruses are characterised generally by their high degree of infectiousness and rapidity of spread. Very minute doses of the virus also are capable of producing a manifest infection. This has been well illustrated under experimental conditions. Thus it has been shown that 0·000001 c.c. of an emulsion of brain tissue from a monkey infected with yellow fever will cause the disease on injection into another animal.

The diseases of man that have been generally accepted as due to filter-passing viruses are smallpox and vaccinia, rabies, epidemic poliomyelitis, encephalitis lethargica and certain other forms of encephalitis, herpes febrilis, herpes zoster, chicken-pox, measles, the common wart, molluscum contagiosum, trachoma, inclusion conjunctivitis, climatic bubo (*lymphogranuloma inguinale*), Rift valley fever, yellow fever, dengue, phlebotomus fever and other three-day fevers of tropical and subtropical countries, psittacosis, mumps, influenza, and the common cold. While in most of these diseases the evidence that the specific etiological factor is a filterable virus has been well established, in some cases it is not altogether conclusive or complete. These questions will be discussed later.

In addition, a number of prevalent animal diseases are due to similar viruses—foot-and-mouth disease, the pox diseases of various animals and birds, distemper of dogs, swine-fever (hog cholera), cattle plague (rinderpest), African horse sickness, fowl-pest, vesicular stomatitis of horses, Borna disease of horses, pseudo-rabies of cattle, louping-ill of sheep, swine influenza, ectromelia of mice, and various others.

A filterable causal agent was also demonstrated by Rous in 1911 in cases of fowl sarcoma, and has given rise to much speculation and discussion regarding the possible virus etiology of malignant tumours. Though certain lesions which might be classified as simple neoplasms (*vide supra*) have been found to

be due to viruses in mammals, no causative virus like that of the Rous sarcoma has been discovered in the malignant tumours of animals other than certain birds.

Levaditi has pointed out that a group of filter-passing viruses, *e.g.* those of rabies, vaccinia, poliomyelitis, encephalitis and herpes, resemble one another not only in their general characters, but also in possessing in common an affinity for ectodermal structures—skin, cornea, etc., on the one hand, and nervous system on the other. He has applied the general term *ectodermoses* to the resulting lesions and regards the viruses as being of two main types, namely, *dermotropic* and *neurotropic*. He has arranged them according to their affinities and regards the poliomyelitis virus as the most strictly neurotropic. The vaccinia virus is at the other extreme of the series, being specially dermotropic, while the virus of herpes occupies an intermediate position, producing lesions both in the skin and central nervous system in experimental animals. It may be mentioned in this connection that, as shown by Marie, the vaccinia virus can be adapted to growth in the cerebral tissue; thereafter this "neuro-vaccine" (Levaditi) can be maintained indefinitely in series, and it then possesses a fixed virulence, as in the case of the virus of rabies. The work of Ledingham especially has shown, however, that cells of epiblastic origin are not those chiefly affected by vaccinia virus, since the lesion is essentially an infective granuloma in which the reticulo-endothelial system is primarily and dominantly involved.

As regards the disease effects produced by neurotropic viruses it must be emphasised that certain filterable viruses under natural conditions produce diseases in man or animals in which the lesions affect chiefly or exclusively the central nervous system. Also susceptible animals when infected with these experimentally by inoculation at sites remote from the brain and spinal cord, as a rule develop similar lesions. Some other viruses, again, show little or no tendency to localise in the central nervous system, but when brought into direct contact, by intradural or intracerebral inoculation they exert pathogenic action locally. To all such viruses the term "neurotropic" has been loosely applied. Hurst has suggested a more satisfactory pathological basis for their classification as follows. (*a*) The *strict neurotropes*, such as poliomyelitis, rabies, pseudorabies (in the monkey) and Borna disease viruses. These attack directly and destroy nerve cells, other lesions being secondary. They reach the central nervous system from a peripheral site of inoculation by the nerves of the part. They

multiply only in nervous tissue and they do not appear in the blood or spinal fluid till the disease is far advanced. Intravenous inoculation usually does not succeed except with large doses. (*b*) The *pantropic viruses*: one type, such as those of herpes febrilis (in the rabbit), pseudo-rabies (in the rabbit), while neurotropic also attacks cells of all the embryonic layers. Neural spread occurs. A second type, represented by yellow fever and louping-ill, is more restricted in its affinities, and the nervous lesions tend to develop late in the disease, after antibodies have appeared in the blood. The latter type is often spread by blood-sucking arthropods. In both types intravenous inoculation with small doses often succeeds. Also there is evidence that the infection spreads by the circulation. (*c*) The *viscerotropic viruses*, such as those of vaccinia and lymphogranuloma inguinale, when inoculated directly into the brain produce primarily meningeal lesions and any damage of nerve cells is secondary. Under natural conditions they do not produce encephalitis.

The Demonstration of Filterable Viruses and Experimental Methods of Studying these Viruses

Rigid precautions must be exercised in attempting to demonstrate such viruses in order to obviate fallacious results. Every filter employed must have been proved to arrest the ordinary bacteria. The filtration should be carried out within the minimum time, but the positive or negative pressure used in filtration should be as slight as possible—50 mm. of mercury, if possible, and never exceeding 500 mm. Fluids rich in protein, *e.g.* blood serum, must be diluted 10- to 50-fold to facilitate their filtration and to avoid clogging of the filter. In dealing with tissue emulsions or inflammatory exudates, before filtration the material should be centrifuged at 3000 r.p.m. for thirty minutes and then passed through asbestos pulp spread over a coarse fritted glass disc [1] The demonstration of the virus will depend on the experimental production of the characteristic disease in susceptible animals (or, in some cases, human volunteers) by inoculation of the filtrate obtained from material likely to contain the virus (*e.g.* blood serum, nasal and throat secretions, tissue emulsions, inflammatory exudates). A convenient method of controlling the permeability of the filter is to add an emulsion of *B. prodigiosus* [2] to the material before filtration and test the filtrate for sterility by the usual culture methods. In any case the bacteriological sterility of the filtrate must be controlled by aerobic and anaerobic cultivation tests. To establish the occurrence of propagation on the part of the virus, passage through a series of susceptible animals is carried out after the first

[1] Obtained from Jena^er Glaswerk, Schott & Gen. Jena.

[2] A small saprophytic bacillus which in culture at room temperature produces a pink pigment, thus rendering the colonies easily recognisable.

experimental results are obtained. In any preliminary experiments, one of the coarser filters is used, *e g.* a Berkefeld V or Chamberland L_2. In further tests, finer filters may be employed to estimate the degree of filterability of the virus With a view to determining the size of virus particles, methods of " ultra-filtration " have been applied in which the filter membrane consists of a thin layer of collodion. According to their mode of preparation the permeability of such membranes may be graded (*vide* p. 716); the permeability also depends on the filtration pressure employed A difficulty which attends the interpretation of the results of such experiments is that the virus may be attached to other particles, and so its retention by the filter may be due to the characters of the latter. The distribution of a virus when a suspension of material containing it is centrifugalised may likewise depend on the size of the particles to which it is attached.

A source of fallacy in the investigation of diseases due to viruses has come to light in recent work Apparently normal animals may harbour a virus which only becomes virulent after repeated passages. Thus " virus III " of rabbits (Rivers and Tillett) was discovered as the result of repeated transfers of testicular material through a series of animals, the original inoculation having been with blood of rabbits which seemed healthy (Andrewes and Miller). In the first passages no changes followed the injections, but in later transfers the intratesticular inoculation produced acute orchitis and pyrexia. Intranuclear cell inclusions were found, especially in the macrophages. Other species of animals were not susceptible. Similarly the salivary glands of guinea-pigs appear to contain frequently a transmissible virus which causes a local mononuclear reaction and the formation of intranuclear inclusion bodies Intratesticular inoculation of the virus causes similar lesions, and injection into the brain leads to acute meningitis.

Staining of Inclusion Bodies.—These are usually acidophile and less frequently basophile. Dilute Giemsa's stain, used as in staining elementary bodies (*vide infra*), is suitable for demonstrating basophile inclusions, *e.g.* in psittacosis. For the acidophile forms several stains have been successfully employed of which the following may be mentioned.

Mann's methyl blue eosin stain.—*Composition:* 1 per cent. aqueous solution of methyl blue, 35 c.c , 1 per cent. aqueous solution of eosin, 45 c.c.; distilled water, 100 c.c. *Method.*—Tissues are fixed in Bouin's or Zenker's solution[1]: the sections are stained for twelve hours at 37° C., rinsed in water and differentiated in 70 per cent. alcohol (to which 5 drops of saturated aqueous Orange G have been added for each 5 c.c. of 70 per cent. alcohol), the differentiation being controlled microscopically; the section is subsequently dehydrated and mounted in the usual way.

Ford's modification of Mann's stain.—Sections are stained as above for three hours at 37° C.; they are then treated with pure formalin for five seconds, washed in water and differentiated as in

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[1] Bouin's solution consists of saturated aqueous picric acid solution 15 c.c., formalin 5 c.c., glacial acetic acid 1 c.c.; mix before use. Fix pieces of tissue according to size for two to twenty-four hours; transfer them directly to several changes of 80 per cent. alcohol. Zenker's solution, see p. 133.

the original process. This method has been found particularly valuable in demonstrating the Negri bodies of rabies.

Staining of Elementary Bodies.—*Giemsa's solution* diluted 1 in 10 with distilled water and applied for twelve to twenty-four hours has been extensively employed The elementary bodies of vaccinia, psittacosis and herpes may all be stained in this way. The principal disadvantages of the method, however, are its slowness and the tendency for the stain to form deposits. Further, different samples of the stain vary considerably in their staining affinities

Paschen's Stain —For speed and reliability this method is unrivalled. Films from infected material are made on slides ; allowed to dry ; placed in distilled water for five minutes , dried in air ; fixed in absolute alcohol for five minutes ; dried in air ; treated with Löffler's flagella mordant (tannic acid, 20 per cent aqueous solution, 100 c c ; ferrous sulphate, saturated solution, 50 c c. ; saturated alcoholic solution of basic fuchsin, 10 c c.) which should be gently heated for one minute and allowed to act for ten minutes ; washed in distilled water and stained for ten minutes with 5 per cent. carbol-fuchsin solution (with the application of heat for one minute). The preparation should thereafter be rapidly washed in water, dried by blotting and mounted in cedar-wood oil

Victoria Blue.—Herzberg has recently drawn attention to the value of this dye for the demonstration of the elementary bodies of vaccinia, varicella, herpes, and canary-pox. Films are made as usual, kept at room temperature for twenty-four hours, placed in the incubator at 37° C. for one hour, stained with a 3 per cent. aqueous solution of Victoria blue for ten minutes ; washed with water, rapidly dried in air and thereafter mounted in balsam. Individual elementary bodies are coloured dark blue and the dye appears to have little affinity for cell cytoplasm.

Castaneda's Method.—This has also been used very successfully for staining *Rickettsiæ*. (*a*) *Buffer Solution* : potassium dihydrogen phosphate, 1 gram in 100 c.c. of distilled water, and sodium monohydrogen phosphate (Na_2HPO_4, $12H_2O$), 25 grams in 900 c c. of distilled water, are mixed together and the pH adjusted to 7·5 1 c.c. formalin is then added as a preservative. (*b*) *Stain* : 100 c.c methyl alcohol and 1 gram of methylene blue are mixed. To 20 c.c. of the buffer solution 1 c.c. of formalin and 0·15 c.c. of the stain are added and this mixture is applied to the film for three minutes and decanted without washing. The preparation is then counterstained for one to four seconds with safranin (Safranin " O," 0·2 per cent. in distilled water, 1 part ; acetic acid, 0·1 per cent., 3 parts). The film is finally washed with running water and dried.

The Use of the Ordinary Microscope for demonstrating Elementary Bodies.—The usefulness of the ordinary microscope for this purpose has been somewhat overlooked. The limit of visibility attainable with white light is stated to be 0·074 μ and with green light 0·0673 μ (Coles). The macula is more sensitive to green light and the additional contrast which is imparted enables smaller objects to be more easily detected. The occurrence of these minute structures is of importance in the recognition of certain virus diseases. Thus, in recent years elementary bodies have been demonstrated in vaccinia, variola, ectromelia, herpes, varicella, psittacosis, fowlpox, canary-pox, infectious myxomatosis

and fibromatosis of rabbits. It must be borne in mind that the employment of mordants for staining elementary bodies increases their size, so that although vaccinia virus may actually measure 0·15 μ, after staining it appears to be 0·3 μ. With improvements in technique it is possible that viruses even smaller than 0·067 μ may be seen as stained particles. Much, however, depends on the skill of the investigator as well as his visual acuity and optical equipment.

Optical Equipment.—The choice of suitable equipment[1] is as important as the correct use of it and strict attention should be paid to the maker's instructions regarding the optimum working conditions of each lens. The best source of illumination has been found to be the intensity lamp with the short low-voltage filament. The lamp is fitted with a condenser and an iris diaphragm for dark-field and critical bright-field illumination, also a ground-glass screen for direct observations.

Before examining a preparation for elementary bodies it is necessary to ensure that the lamp filament, substage condenser, objective, and eyepiece are accurately centred and so in perfect alignment; with particles less than 0·3 μ in size this is imperative. Stained films should be made on slides 1·1 mm. thick and mounted preferably in cedar-wood oil. It is also advantageous to place the mounted slide between two heavy weights for twenty-four hours in order to bring the coverslip and slide into close apposition. The preparation should be viewed first by direct light with the low power dry objective to ascertain the types of cells present, and then a 2 mm. oil immersion objective with × 10 and × 15 oculars can be used to detect elementary bodies Fields showing only scanty elementary bodies should be disregarded and if possible clumps of them should be found. Individual bodies or pairs should be moved into the centre of the field and carefully scrutinized first with the ×10 and then the × 15 ocular; the fine adjustment should be continuously used and the height of the substage condenser varied in order to focus accurately the light upon individual particles. The diaphragm may also be slightly closed in order to give greater depth of focus and a green filter may be used with benefit in order to impart greater contrast to the particles. Each stained film made from pathological material which is to be examined for elementary bodies, should be accompanied if possible by two control specimens—one from an identical source of normal tissue and the other from a specimen which is known to contain elementary bodies. All three slides should be stained together and examined simultaneously. Unless these controls are included in every series, erroneous conclusions are likely to ensue. The staining reactions

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[1] The following apparatus has been found useful (van Rooyen): Patna microscope (Watson) fitted with rackwork draw-tube, universal mechanical stage and centring substage mechanism; compensating eyepieces × 10, × 15, and × 20 (Zeiss); apochromatic objectives, 2 mm. (1/12 in. oil immersion) of N.A. 1 3 (Zeiss), or 2 mm. holoscopic objective of N.A. 1 37 (Watson); 8 mm. dry apochromatic objective of N.A. 0·65 (Zeiss); and 6L dry achromatic objective of N.A. 0·65 (Leitz). The most suitable substage condensers have been found to be either Watson's parachromatic dry condenser of N.A. 1 or, better, the holoscopic oil immersion model of N.A. 1·3 or 1·7. With the latter, however, the slides must not exceed 1·3 mm. in thickness.

and morphology of any virus bodies having been noted, the same field should next be examined by dark-ground illumination. This can be done by turning the 2 mm. objective off the slide (which should be firmly fixed to the microscope stage with clips), wiping the oil from the surface of the coverslip with a piece of muslin, and then turning either the 6L or 8 mm [1] lens into position The objective should next be focussed upon the slide, the ground glass lamp screen removed, both iris diaphragms fully opened, and a patch stop or a Travis's expanding stop inserted into position in the condenser substage ring Dark-ground illumination is now obtained and it should be possible to determine whether or not the stained elementary bodies previously seen in the field are also refractile to obliquely transmitted light. By reversing the steps detailed above it is possible to restore the direct illumination of the same field. For further details of the method, see van Rooyen (1937).

Cultivation of Viruses.—Several procedures have yielded cultures.

Cultivation in the Chorio-allantoic Membrane of the Developing Chick Embryo. Since Goodpasture, Woodruff and Buddingh demonstrated that the viruses of fowlpox and vaccinia were readily cultivable in the chorio-allantoic membrane of the developing chick this method has been extensively employed for other viruses, *e.g.* those of ectromelia, psittacosis, and influenza. The following method can be recommended. Fertile hens' eggs are washed with soap and water, placed in an egg incubator at 41° C for eight to ten days and then " candled " before a 60 c p. electric lamp in order to determine whether development of the embryo has occurred. The latter is indicated by a dense shadow situated at one part of the egg, with a clear semi-transparent patch lying immediately adjacent to it The shell is next gently rotated before the light until the clear zone occupies the top half of the shell and a pencil mark made at the uppermost point of this area. At the same time the position of the air sac is noted and a second pencil mark likewise made above this cavity The surface of the egg is now thoroughly washed and scrubbed in lukewarm water (41° C.) containing soap to which 2 per cent. phenol solution has been added. Prior to inoculation further sterilisation of the shell is carried out with 4 per cent. iodine in spirit. During the above procedure the egg is placed upon a ring of sterile plasticine and handled with aseptic precautions. The method of choice for inoculation of the egg depends upon the bulk of the inoculum. If this does not exceed 0·5 c.c. it is practicable to puncture the shell with the point of a sharp pair of scissors and then introduce the material beneath the shell membrane with a syringe to a vertical depth of 3 mm., so that the needle point rests upon, but does not penetrate, the chorio-allantoic membrane beneath. Should it be desired, however, to inoculate more than 0·5 c.c. of fluid it is advisable first to puncture the shell over the air sac before inoculating the egg as previously described. In this way it is possible to secure greater accommodation within the shell for the increased bulk of inoculum. Occasionally when embryos of ten to twelve days old are used, slight hæmorrhage may occur on puncturing the shell membrane, but the mortality from this is negligible. Both apertures in the shell

[1] See footnote on p. 726.

are sealed with sterile tissue paper and paraffin and the egg is thereafter incubated at 37° C., preferably in an incubator fitted with a large trough containing water. After three or four days the eggs are removed from the incubator, the paraffin seal scraped off the shell, which is then painted with 4 per cent. iodine and opened under aseptic conditions.

The appearances observed in the chorio-allantoic membrane vary with the individual virus cultivated. For example, if vaccinia virus is grown the embryo is frequently dead and large white opaque discs or "plaques" measuring from 3 to 4 mm in diameter, which may be either discrete or confluent, are visible on the surface of the membrane. The infected tissue is then resected with a sharp pair of scissors, tested for the presence of bacterial contamination both aerobically and anaerobically by the usual cultural methods, and if found to be free from cultivable bacteria, examined for pathogenicity by inoculation of a susceptible animal.

Cultivation in vitro *in Minced Chick Embryo*.—This method has been successfully used for the cultivation of the viruses of influenza, Rift Valley fever and lymphogranuloma inguinale. Eight-days-old embryos are withdrawn from carefully sterilised egg shells with aseptic precautions and then finely minced in a sterile glass receptacle with a sharp pair of scissors. About 0·2 c.c of the semi-solid mass of embryonic tissue is next withdrawn with a wide-bore tumour syringe and placed in a sterile Carrel flask. A few drops of the virus material are added to the mass and allowed to permeate the tissue and 5–10 c.c. of Tyrode's solution are added. Cultures are incubated at 37° C. for four days and examined microscopically for proliferation of mesenchyme cells and by animal inoculation for the growth of the virus.

Tyrode's solution is composed as follows: 1000 c.c. distilled water, sodium chloride 8 grams, potassium chloride 0·2 gram, dextrose 1 gram, sodium dihydrogen phosphate 0·05 gram, sodium carbonate 1 gram, magnesium chloride (5 per cent. solution of the anhydrous salt) 4 c.c, calcium chloride (5 per cent. solution of the anhydrous salt) 4 c c. The solution, which is strongly alkaline, is adjusted to pH 7·5 by addition of 5 per cent. solution of phosphoric acid (sp. gr. 1·75). Over acidity is corrected with 10 per cent. sodium carbonate solution. The final solution is sterilised by filtration at a pressure of 30 mm. of mercury through a Seitz (EK) filter. The solution may then be divided aseptically into smaller volumes and stored in the ice-chest until required. Only pure chemicals and freshly distilled water in perfectly clean glass vessels should be used for the preparation of Tyrode's solution.

Cultivation in the presence of surviving tissue cells (Maitland and Maitland). The cultivation of vaccinia virus in a medium containing the fresh kidney tissue of the rabbit or hen is described later (*vide* p. 734).

Cultivation in tissue cultures.—Growths of epithelia, and fibroblast cells have been proved to be susceptible to infection by certain viruses. The methods employed are too complicated for detailed description here and the reader is referred to the original literature on this subject (Bland and Canti; Rivers, Haagen and Muckenfuss; Rhodes and van Rooyen).

Preservation of Viruses.—Certain viruses present in animal tissues can be preserved for considerable periods in 40 per cent.

glycerol made up in physiological saline. The most effective method is rapid desiccation as in the preservation of complement (*vide* p. 150) and maintenance *in vacuo* at low temperatures The tissue should be finely divided by mincing with scissors before desiccation

Smallpox (Variola) and Vaccinia

Smallpox is a disease to which much study has been devoted owing, on the one hand, to the havoc which it formerly wrought in Europe, and, on the other hand, to the controversies which have arisen in connection with the active immunisation against it introduced by Jenner. It has been recognised for some time now that the *contagium vivum* present in the disease is not one of the ordinary micro-organisms but is of more minute size and classifiable with the filterable viruses. In the unmodified disease mixed infections, especially with streptococci, tend to occur, and this contributes to the serious results.

Jennerian Vaccination.—Until Jenner's time the only means adopted to mitigate the disease had been by inoculation (by scarification) of virus taken from a smallpox pustule, especially from a mild case ; by this means a mild form of the disease was often originated. It had previously been known that one attack of the disease protected against future infection, and that the mild attack produced by inoculation also had this effect. This inoculation method had long been practised in various parts of the world, and had considerable popularity all over Europe during the eighteenth century. Its disadvantage was that the resulting disease, though mild, was still infectious, and thus might be the starting-point of a virulent form among unprotected persons. Jenner's discovery was published when inoculation was still considerably practised. It was founded on the popular belief that those who had contracted cowpox from an affected animal were insusceptible to subsequent infection from smallpox. In the horse there occurs a disease known as horsepox, especially tending to arise in wet, cold springs, which consists in an inflammatory condition with vesicle formation about the fetlocks, giving rise to ulceration. Jenner believed that the matter from these ulcers, when transferred by the hands of those who dressed the sores to the teats of cows subsequently milked by them, gave rise to cowpox in the latter. This disease was thus, in his opinion, identical with horsepox, and it had its origin in epidemics of the latter. Cowpox manifests itself as a papular eruption on the teats ; the papules become pustules ; their contents dry up to form scabs, or more

or less deep ulcers at their sites. From such a lesion the hands of the milkers may become infected through abrasions, and a similar local eruption occurs, with general symptoms in the form of slight fever and malaise. It is this illness which, according to Jenner, gave rise to immunity from smallpox infection. He showed experimentally that persons who had suffered from such attacks did not react to inoculation with smallpox; and further, that persons to whom he communicated cowpox artificially were similarly immune. The results of Jenner's observations and experiments were published in 1798 under the title, *An Inquiry into the Causes and Effects of the Variolæ Vaccinæ*. Though from the first, Jennerian vaccination had many opponents, it gradually gained the confidence of the unprejudiced, and became extensively practised all over the world, as it is at the present day.

The so-called vaccine lymph which contains the immunising agent is the serous exudate of the cowpox vesicle. When such lymph is used for inoculating calf from calf by *passage* a continuous supply of a product of very constant potency is obtained; and the lymph used for human vaccination is produced by artificial inoculation of these animals. The virus is also maintained in rabbits to serve as "seed lymph" for inoculating calves. By the use of this vaccine immunity against smallpox is conferred on the vaccinated individual for a considerable period. There has been some doubt whether strains of the cowpox or vaccinia virus in use for the production of the vaccine originate from natural cowpox or represent the actual variola virus modified by passage in calves and rabbits. Many of such strains are probably variolous in origin. The relationship of the variola and vaccinia viruses will be discussed later. The most striking evidence in favour of vaccination is derived from its effects among the staffs of smallpox hospitals; for here, in numerous instances, it is only the unvaccinated individuals who have contracted the disease. While vaccination is undoubtedly efficacious in protecting against smallpox, Jenner over-estimated the duration of the immunity. It has been noted in smallpox epidemics that whereas young unprotected subjects readily contract the disease, those vaccinated as infants escape more or less till after the thirteenth to the fifteenth years. Revaccination is therefore necessary if immunity is to continue; and where this is done in any population, smallpox becomes a rare disease, and the mortality is practically nil. The whole question of the efficacy of vaccination was investigated in this country in 1896 by a Royal Com-

mission, whose general conclusions were as follows. Vaccination diminishes the liability to attack by smallpox, and when the latter does occur, the disease is milder and less fatal. Protection against attack is greatest during nine or ten years after vaccination. It is still efficacious for a further period of five years, and possibly never wholly ceases. The power of vaccination to modify an attack outlasts its power wholly to ward it off. Revaccination restores protection, but this operation must be from time to time repeated. Vaccination is effective according to the thoroughness with which it is performed.

The Relationship of Smallpox (Variola) to Cowpox (Vaccinia).—This is a question regarding which great controversy has taken place ; a subsidiary point has been the inter-relationships within the group of animal diseases, which includes cowpox, horsepox, sheeppox, etc. There is no doubt that cowpox can be communicated to man, in whom it produces the vesicle limited to the point of inoculation and the slight general symptoms which vaccination with calf lymph has made familiar. On the other hand, it does not reproduce in man a general eruption, and the local eruption is only infectious when matter from it is introduced into an abrasion. This loss of infectiousness by transmission through the body of a relatively insusceptible animal is a condition which is known in other diseases. Monkeys are susceptible to both vaccinia and variola. On inoculating the shaved skin by scarification a papulo-vesicular lesion results in each case, and the lesion can be indefinitely maintained in series. Further, monkeys thus inoculated with lymph from vaccinia are rendered immune against variola, and the converse holds ; though Gordon found that the degree of protection produced by vaccinia against variola was rather greater than that produced by variola against vaccinia. In the rabbit, on the other hand, similar inoculation with vaccinia gives rise to the typical lesion, whereas inoculation with variola produces only slight effects. An even more delicate method than inoculation of the skin for detecting the action of vaccinia in the rabbit is to inject the material into the testis, an orchitis being set up in consequence. The calf is not highly susceptible to variola, and the typical lesions are not produced at first. A slight reaction may occur at the site of inoculation, and if inoculation is made from the lesion to another calf, and then continued in series, the lesions may gradually become more marked and ultimately assume the characters of those produced by vaccinia. Thereafter, the altered virus may be

indefinitely transmitted. This result has been repeatedly obtained, and the animals thus treated become immune against natural vaccinia. Not only so, but on using for human vaccination the lymph from such calves, results indistinguishable from those produced by vaccine lymph are obtained, and the transitory illness which follows, unlike that produced in man by inoculation with smallpox lymph, is no longer infectious. In fact, some of the strains of vaccine virus originated from the variolation of calves. Thus the facts available go to show not only that vaccinia confers immunity against variola, but that variola confers immunity against vaccinia. All the evidence in regard to variola and vaccinia indicates that these diseases are due to the same virus, and that the differences between them result from the relative susceptibilities of the two species of animals in which they occur naturally, and the modifications in virulence produced by habitat in different species.

As has been noted above, vaccinia when inoculated into the skin in man produces only a local lesion ; a similar inoculation in susceptible animals leads to the same result. But Camus has shown that in rabbits the intravenous injection of vaccine lymph in sufficient doses may produce generalised lesions, and this has been confirmed by others. Further, McIntosh has found that cutaneous inoculation in the rabbit with a strain of vaccine virus rendered highly virulent by passage produced frequently a general eruption with internal focal lesions. In the human subject also, after vaccination which runs the ordinary course, the virus may be present in the blood. Therefore, in view of the fact that such dissemination of the virus may occur, the question has arisen whether the encephalitis which has been observed occasionally to follow vaccination is caused by vaccinia (p. 761).

Cowpox virus produces lesions in most mammalia and also in fowls and pigeons. With regard to the relation of cowpox to horsepox, it is probable that they are the same disease. Both are characterised by localised eruptions, and both appear to be ordinarily transmitted by human agency. Some outbreaks of cowpox have originated from the horse, but in other cases such a source has not been traced. Sheeppox, which occurs chiefly in south-eastern Europe, is an interesting affection, as it presents certain analogies to human smallpox. It is a highly infectious disease, characterised by a generalised eruption. Inoculation of the sheep with lymph from a vesicle usually produces only a local lesion (though sometimes there is a slight general eruption), and thereafter the animal is immune. Inoculation of herds is carried out as a preventive measure and is effective, but the animals thus treated are still infectious to fresh animals. Goatpox presents similarities to sheeppox, but goats do not become infected during outbreaks of

sheeppox, and lymph from the latter is practically without effect on the goat. Swinepox, a disease also with a disseminated eruption, is rare; infection is said to come from vaccinia. It has been generally concluded from these and other observations that there are several varieties of viruses, but that the two main types are those of cowpox and sheeppox. Gins, however, by means of passage through rabbits, succeeded in transforming sheeppox and goatpox, as well as swinepox, into cowpox; and accordingly the different viruses are apparently to be regarded as modifications of the same.

Fowlpox is an analogous disease which occurs in birds; but the evidence suggests that the virus of this infection is distinct from those of the mammalian pox diseases

Alastrim.—During recent years much attention has been directed to a variola-like disease which is characterised by the relative absence of constitutional disturbances and by its non-fatal character, whilst the skin lesions may be severe, sometimes of a confluent type. It is highly infectious, and epidemics have occurred practically all over the world. Various names have been applied, such as para-variola, mild smallpox, and alastrim; the last term has now come into general use. There has been much controversy with regard to its relation to variola, but we can refer only to the experimental work. The results of inoculation of animals are closely similar to those in the case of variola. In monkeys the lesions are the same with the two viruses, though Gordon found that those of the alastrim virus were rather less severe. As in the case of variola, rabbits and calves are resistant to the alastrim virus or only slight lesions occur, but Blaxall found that on passage through the calf the alastrim virus became so modified that it produced the typical lesions of vaccinia. Immunity studies have brought out no marked differences. Vaccinia protects against alastrim as it does against variola, though differences in degree may be noted, and Gordon has found that the antibodies in anti-vaccinia serum react also with the alastrim virus. Cross-immunity tests, however, indicate that there is some antigenic difference between the vaccinia and alastrim viruses; thus, though vaccination protects against alastrim, cases of the latter disease have frequently been found susceptible to vaccinia on vaccination. All the evidence at present available goes to show that the alastrim virus represents a modified form or variant of the variola virus, the modification consisting mainly in the loss of toxic and lethal properties towards the human subject, while pathogenic action in animal tests and antigenic properties have been little altered.

Nature of Virus.—It has been conclusively shown that the

virus of variola and of vaccinia is not one of the ordinary bacteria. In the lymph of skin lesions bacteria are, of course, present, but these can be removed or killed without the infective properties of the lymph being affected. Noguchi, for example, obtained this result by passage through the testes of rabbits. The filtration of vaccine lymph through a Berkefeld filter has given rather divergent results. Sometimes the filtrate is infective, sometimes not. The result apparently depends upon the amount of adsorption which occurs in the pores of the filter, as it has been shown that substances like kaolin, animal charcoal, phosphate of calcium, etc., adsorb the vaccinia virus and others of similar nature. Ward has found that the virus is most readily filterable in " hormone " broth with a pH of 7·6. Gordon observed that the virus is affected by gravity and is largely thrown down on centrifuging, and concluded that it is of particulate character. Very minute coccoid bodies have been demonstrated by Paschen and others in infective lymph and also in lesions, by dark-ground illumination and other methods, and many observers now hold the view that these " Paschen bodies " represent the actual virus (*vide infra*). It is generally accepted that the virus belongs to the filter-passing group, and it has been estimated by filtration through graded collodion membranes (Elford) that the virus particles are from 0·125 to 0·175 μ in diameter. Although the virus from variola has not yet been cultivated outside the body, that from vaccinia has been found to multiply in a medium consisting of fresh minced adult hen or rabbit kidney along with fresh serum and Tyrode's solution (p. 728) (H. B. and M. C. Maitland). Thus growth *in vitro* can be obtained in the presence of surviving tissue cells. The procedure adopted was to mix bacteria-free vaccine virus with 0·3 c.c. of the minced tissue, and then add 3 c.c. fresh rabbit serum and sufficient Tyrode's solution to make up the volume to 10 c.c. The material was distributed in 2 c.c. quantities in Carrel flasks and incubated. It may be noted that the Paschen bodies appear in these cultures. Cultures can also be obtained readily in the chorio-allantoic membrane of the developing chick and in minced chick embryo (p. 727).

The possibility of vaccinating against smallpox by means of a vaccine virus cultivated *in vitro* in minced chick embryo has been explored by Rivers and Ward, the virus being injected intradermally. The reactions do not lead to an open sore or scarring as in Jennerian vaccination. This method calls for further investigation ; at present no statement can be made regarding its effectiveness in

preventing smallpox though such inoculation is capable of preventing a later infection with the same virus from calf lymph.

The virus is comparatively thermolabile, its pathogenic properties being practically destroyed on exposure for an hour at 55° C. though it has still some antigenic power (Gordon). It is readily destroyed by various antiseptics, but in this respect its behaviour corresponds to other filterable viruses rather than to bacteria. For example, it persists practically unchanged in 50 per cent. glycerol and in 10 per cent. ether, its reistance to the former being taken advantage of in the preparation of vaccine lymph for preventive inoculation. Gordon has found that the virus both in vaccine lymph and in variolous material is very susceptible to the action of potassium permanganate, being readily destroyed by a solution of 1 : 10,000. In fact, this substance is viricidal in higher dilutions than is carbolic acid or even corrosive sublimate. The virus when dry, as in variolous crusts, remains infective for long periods.

Inclusion and elementary bodies.—Certain peculiar structures are to be found within the epithelial cells of the smallpox and vaccinia lesions both in the natural disease and in that experimentally produced, *e.g.* in the cornea of the rabbit, which is a suitable site for study. They have been specially investigated by Guarnieri, Councilman, Ewing, Prowazek, and others, but their nature is still a matter of dispute. They are usually globoid, measuring about 1–4 μ in diameter, though they may be smaller; sickle-shaped and other forms are met with. They are contained within the epithelial cells, often lying near the nuclei. Supposed dividing and budding forms have been described, and even a cycle of development. There is no doubt that these structures, or "Guarnieri bodies" as they are frequently called, are a feature of the lesions, but there is no evidence that they are protozoal, as some have supposed. They appear to belong to the same class as the inclusion bodies occurring in other virus diseases. With Mann's stain they tend to be acidophile; with certain other stains basophile forms are observed. In variola intranuclear acidophile bodies have also been found, but these seem to be absent from the lesions in vaccinia.

Reference has already been made to the "elementary bodies" of Paschen [1] which are found in the lesions and exudate of smallpox and vaccinia. These are rounded coccoid structures about 0·2 μ in diameter and are demonstrable by Paschen's stain or by prolonged staining with Giemsa's solution which colours them red or

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[1] Minute bodies corresponding to the Paschen bodies were described and illustrated in the exudate of variola by Buist of Edinburgh in 1886. The measurement he gave of these was 0 15 μ and he demonstrated them by prolonged staining with gentian violet. He regarded these minute bodies as "spores" of micrococci which he cultivated from the same material but considered them to be the causal micro-organism of variola and vaccinia. There can be little doubt that the minute organisms described by Buist were the forms which are now designated Paschen Bodies (J. B. Buist, *Vaccinia and Variola*, London, 1887).

purple (Fig. 171). It is possible that staining gives them artificially the appearance of being larger than they really are, and this is suggested by comparisons with photographs taken by ultra-violet light (Barnard) and estimates of the size of the virus particles by filtration through collodion membranes. These bodies can be readily concentrated by high-speed centrifuging (*e.g.* 15,000 revolutions per minute) and by this means have been obtained in an almost pure state. From the centrifuged deposit a suspension of the bodies can be prepared which yields a specific agglutination reaction with an anti-vaccinial serum, behaving like a bacterial suspension when acted on by a homologous antiserum (Ledingham). According to Goodpasture the Guarnieri inclusion bodies consist of aggregates of elementary bodies.

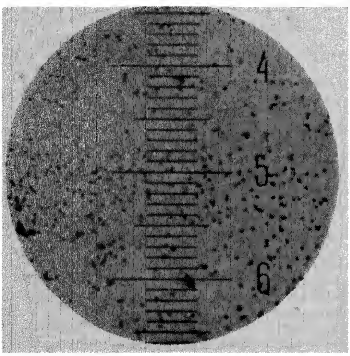


Fig. 171a.—Film from culture of vaccina virus in chorio-allantoic membrane showing elementary bodies stained by Paschen's method; each division of scale $=1 \cdot 14\ \mu$. $\times 1187$.

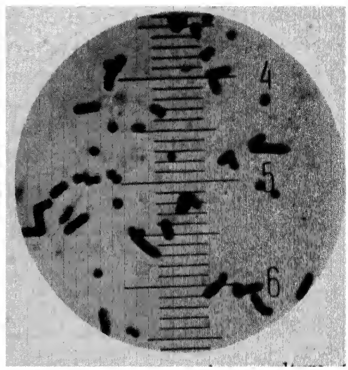


Fig. 171b. — Film from culture of staphylococcus and B. coli mixed. Stained by same method as A and photographed with same magnification for comparison.

From photographs by Dr. C. E. van Rooyen.

Immunity and Antibodies.—Various facts have been mentioned above with regard to protection afforded by cutaneous inoculation, but it has been shown that immunity can also be produced in animals by introducing the virus of vaccinia by various routes—subcutaneous, intravenous, etc. Gordon has studied further the passage of the virus through intact surfaces, and has found that the nasal mucosa is the most permeable, a catarrhal condition resulting which is attended by immunity to cutaneous inoculation. (This observation is manifestly of importance in relation to the possibility that infection in smallpox takes place through the respiratory passages.) In animals immunised by the above methods, antibodies appear in the blood of a nature corresponding in some respects to those recognised in bacterial infection—substances which inactivate

or neutralise the virus (designated " viricidal "), also flocculating and complement-fixing bodies. The presence of the first variety is shown by the fact that when the virus is exposed for some time to the antiserum it is bereft of its infective properties, as indicated by the loss of power to cause lesions on inoculation into the skin. It has been suggested that the union of antigen and antibody is capable of readily undergoing dissociation, since neutral mixtures of vaccinia virus and immune serum which have been kept for some time may become virulent again on dilution (Andrewes). Such apparent dissociation on dilution depends on the relative quantities of antibody and virus; in the presence of a sufficient excess of antibody reactivation of the virus does not occur when the mixture is diluted (Goyal). It is manifestly difficult to determine whether any direct viricidal or even inactivating effect occurs *in vitro* or results only when the mixture of virus and antiserum is introduced into the tissues. It may be noted that serum heated at 55° C. is as active as fresh serum; thus if *in vitro* inactivation occurs, complement does not play any part in it. Fairbrother has shown that when mixtures of vaccinia virus and antiserum are injected intradermally neutralisation can be demonstrated irrespective of the time they have been in contact *in vitro*; this is not so on intracerebral injection, four hours contact being necessary. He suggests that the antibody sensitises the virus to the destructive action of the tissue cells. The actual mechanism of protection by an antiviral serum and its specific antibody remains obscure. Whether or not inactivation of the virus depends on an interaction *in vitro* between virus and antibody, such interaction is manifested by specific flocculation and complement-fixation phenomena. The antibodies in anti-vaccinia serum give flocculating and complement-fixing reactions with the viruses of variola and alastrim as well as with that of vaccinia. It may also be added that corresponding observations have been made on the sera of patients who have recovered from smallpox. It has been found that such sera give complement-fixation not only with material from the lesions of variola but also with the virus of vaccinia. Further, Schneider showed that the serum of variola convalescents had a distinct anti-infective action toward vaccinia virus. Phenomena of supersensitiveness have been observed on re-inoculating with virus, and here also the action of the two types is reciprocal. All these facts support the view already expressed that vaccinia virus is simply the virus of variola modified in certain of its properties by passage through the bovine species. (We may

state that the virus obtained from the lesions of varicella, chicken-pox, gives no reactions with an anti-vaccinia serum, and that no cross immunity phenomena between the two viruses have been observed. It is generally accepted that varicella is quite a distinct disease.) There is no evidence that killed vaccinia virus is capable of producing immunity. It has been suggested that immunity may depend on the persistence of the virus (Douglas, Smith and Price). Thus Olitski and Long were able to obtain the virus from the testes of immune rabbits by a method of concentration by cataphoresis long after recovery from cutaneous inoculation. On the other hand, those animals from which the virus could not be obtained were found to be no longer resistant to re-inoculation.

For the differentiation of variola and varicella some value attaches to the results produced on inoculation of the rabbit's cornea with the contents of the skin lesions (Paul). The cornea is lightly scarified and the material (which if previously dry is rubbed up in a drop of saline) introduced into it. After thirty-six to forty-eight hours if the material has been derived from a case of variola, small, clear, bubble-like elevations have developed on the cornea, which are best seen as opaque white spots when the eye is enucleated (the cornea being then cleansed of any adherent blood or secretions) and placed for a short time —about a minute—in a mixture of 2 parts saturated watery solution of corrosive sublimate with 1 part of absolute alcohol. Vaccinia causes an appearance which is similar, but more marked; but varicella and other infections produce nothing likely to be mistaken for it. The value of this procedure has been verified by Ungermann and Zuelzer and others. The work also of Gordon and of Burgess, Craigie, and Tulloch on the flocculating action of an antivaccinial serum obtained from rabbits when mixed with extracts of the skin crusts from cases of variola and alastrim, indicates that this is a specific test which possesses considerable value for distinguishing these conditions from varicella; extracts of the crusts in varicella yield a negative reaction.

Foot-and-Mouth Disease

This was the first animal disease in which a filterable infective agent was demonstrated. In 1898 Löffler and Frosch found that the fluid from the vesicular lesions of the disease, even after filtration through an earthenware filter, was still infective on experimental inoculation. The disease, which is highly infectious, attacks cattle, sheep, goats, and pigs, and exceptionally certain other animals. The characteristic manifestation of the infection

is an eruption of vesicles on the feet and in the mouth. In this country the mortality is low. Cases have been recorded of its transmission to the human subject by contact with infected animals and from cow's milk.

The disease is easily reproduced experimentally in cattle, sheep, and pigs by applying infective material, *e g* vesicle fluid, to a scarified area of mucous membrane on the lip or mouth. The fluid is infective even after filtration through fine filters, and the virus particles are exceedingly minute. Their estimated diameter is about 10–20 $m\mu$. The virus is also present in the saliva, milk, and urine. The guinea-pig can be successfully infected by dermal inoculation in the pads of the feet. After one to four days the animal develops pyrexia and a vesicular eruption appears on the inoculated foot, followed by vesicles on the other feet and on the mouth. Recovery takes place in most cases, and the animal is immune to subsequent inoculation with the same strain of virus. Rabbits, rats, and mice can also be infected experimentally, but in them the disease is less characteristic.

The virus has been cultivated in tissue cultures made from the pads, lips, tongue, and skin of embryo guinea-pigs. It is rapidly destroyed at 53° C , but is resistant to desiccation Three immunological types of the virus have been defined, designated O (Oise), A (Allemand), and C. Infection with one of these does not confer an effective immunity against the others. Intermediate types have also been described and there is some uncertainty whether the three recognised types are fixed or stable in their antigenic characters. Immunity following infection may last for twelve months, and the serum of an immune animal has the property of inactivating the corresponding type of virus. It has been shown that artificial immunity can be produced by the administration of formolised virus material, but so far effective practical prophylaxis by immunisation has not been established.

Reference has been made above to the extremely infectious nature of the disease, and the mode of spread has been the subject of much study. The infection is readily transmitted by direct contact, but many outbreaks occur whose origin cannot be explained in this way. The survival of the virus in the dry state in discharges, fodder and carcases may explain some outbreaks, but the question has arisen whether the disease is transmitted by healthy animal carriers and even by man. It has also been suggested that wild rodents may be responsible for outbreaks in cattle, and the infection has been demonstrated in hedgehogs. It is still difficult to make any definite statement regarding the various possible sources of infection (see *Reports, Foot-and-Mouth Disease Research Committee*, **1927, 1928, 1931** and **1937**).

Rabies or Hydrophobia

Introductory.—Rabies or hydrophobia is an infectious disease which in nature occurs epizootically chiefly among the carnivora, especially in the dog and the wolf. Infection is carried by the bite of a rabid animal or by a wound or abrasion being licked by such. The disease can be transferred to other species, and

when once started can be spread from individual to individual by the same paths of infection. Thus it occurs from time to time in cattle, sheep, pigs, horses and deer, and can be communicated to man. Cases of infection from man to man by bites are recorded, but the saliva in man does not appear to be so infectious as in dogs. It is to be noted that the virus is extremely potent, as cases of infection taking place through an unabraded mucous membrane by the licking of a rabid animal are on record, and the experimental application of the virus to such surfaces as the mucous membrane of the nose or the conjunctiva is often followed by infection.

In Western Europe the disease is most frequently observed in the dog; but in Eastern Europe, especially in Russia, epidemics among wolves constitute a serious danger both to other animals and to man. Two varieties of rabies are recognised—(1) rabies proper, or furious rabies (*la rage vraie, la rage furieuse; die rasende Wuth*); and (2) dumb madness or paralytic rabies (*la rage mue; die stille Wuth*). The disease, however, is essentially the same in both cases. In the dog the furious form is the more common. After a period of incubation of from three to six weeks, the first symptom noticed is a change in the animal's aspect; it becomes restless, it snaps at anything which it touches, and tears up and swallows unwonted objects; it has a peculiar, high-toned bark. Spasms of the throat muscles appear, especially in swallowing, and there is abundant secretion of saliva; its supposed special fear of water is due to difficulty in swallowing. Gradually convulsions, paralysis, and coma come on; and death supervenes usually about five days after the appearance of symptoms. In the paralytic form, the early symptoms are the same, but paralysis appears sooner. The lower jaw of the animal drops, from implication of the elevator muscles, all the muscles of the body become more or less weakened, and death ensues without any very marked irritative symptoms.

In man the incubation period after infection varies from fifteen days to seven or eight months, or even longer, but is usually about forty days. When symptoms of rabies are about to appear, certain prodromata, such as pains in the wound and along the nerves of the limb in which the wound has been received, may occur. This is succeeded by a stage of nervous irritability, during which all the reflexes are augmented. There are spasms, especially of the muscles of deglutition and respiration, and cortical irritation indicated by delirium may occur. On this follows a period in which all the reflexes are diminished, weakness and paralysis are observed, convulsions occur, and finally coma and death supervene. The duration of the acute illness is usually from four to eight days, and death invariably results. Rabies in man sometimes assumes a paralytic form.

This is usually manifested at first by paralysis of the limb in which the infection has been received, and of the neighbouring parts, and then the occurrence of widespread and progressive paralysis is the outstanding feature. In man there occur cases where the cerebellum and also the sympathetic system seem to be specially affected.

A peculiar outbreak of rabies in Trinidad, taking the form of an acute ascending myelitis, was studied by Hurst and Pawan. Their observations indicated that the infection originated from cattle, in which the disease was present at the same time, and was transmitted by blood-sucking vampire bats. A similar type of rabies infection in animals has been reported from Brazil.

The Pathology of Rabies.—In rabies the appearances discoverable by an ordinary examination of the nervous system, to which all symptoms are naturally referred, are comparatively unimportant. On naked-eye examination, congestion, and, it may be, minute hæmorrhages, especially in the medulla, are the only features noticeable. Microscopically, lymphocytic exudation into the perivascular lymphatic spaces in the nerve centres has been observed, and in the nerve cells various degenerations have been described. Round the nerve cells in the grey matter of the medulla and cord, Babés described accumulations of newly formed cells, and Van Gehuchten observed a phagocytosis of the nerve cells in the posterior root ganglia and also in the sympathetic ganglia. A most important feature, however, is the presence of the structures known as Negri bodies in the nerve cells (p. 743). In the white matter, especially in the posterior columns, swelling of the axis cylinders and breaking up of the myelin sheaths have been noted, and similar changes may occur also in the spinal nerves, especially of the part of the body through which infection has come. The changes in the other parts of the body are unimportant.

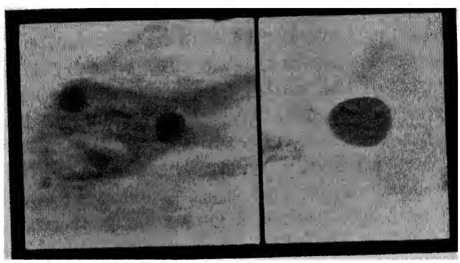
Pasteur's first contribution to the pathology of rabies was to show that the most certain method of inoculation was by inserting the infective matter beneath the dura mater. He found that in the case of any animal or man dead of the disease, injection by this method of emulsions of any part of the central nervous system, of the cerebro-spinal fluid, or of the saliva, invariably gave rise to rabies, and also that the natural period of incubation was shortened. Further, the identity of the furious and paralytic forms was proved, as sometimes the one, sometimes the other, was produced, no matter which form had been present in the original case. Inoculation into the anterior chamber of the eye is nearly as efficacious as subdural infection,

and so also is injection into a peripheral nerve, *e.g.* the sciatic. Intramuscular injection into the muscles of the neck leads to infection in a high proportion of cases. Subcutaneous or intravenous injection may or may not give rise to the disease ; while intraperitoneal inoculation is very uncertain.

In consequence of the introduction of these inoculation methods, further information has been acquired regarding the spread and distribution of the virus in the body. Gaining entrance by the infected wound, it early manifests its affinity for the nervous tissues. It reaches the central nervous system chiefly by spreading up the peripheral nerves. This can be shown by inoculating an animal subcutaneously in one of its limbs with virulent material. If now the animal be killed before symptoms have manifested themselves, rabies can be produced by subdural inoculation from the nerves of the limb which was injected. Further, rabies can often be produced from such a case by subdural inoculation with the part of the spinal cord into which these nerves pass, while the other parts of the animal's nervous system do not give rise to the disease. This explains how the initial symptoms of the disease (pains along nerves, paralysis, etc.) so often appear in the affected part of the body, and it probably also explains the fact that bites in such richly nervous parts as the face and head are much more likely to be followed by rabies than bites in other parts of the body. The virus seems to have a selective affinity for the salivary glands, as well as for the nervous system. Roux and Nocard found that the saliva of the dog became infective three days before the first appearance of symptoms of the disease. The blood is usually non-infective, as are also the solid organs except certain glands, *e.g.* the pancreas.

The Virus of Rabies.—The causal agent of rabies is extremely small, as it can pass through the coarser Berkefeld filters and also sometimes through the coarser Chamberland filters. This is shown by the fact that if an emulsion of any infective material (*e.g.* the brain) be thus filtered, the filtrate is also infective. Evidence that it is the virus itself which passes through is found in the fact that when an animal dies on injection with the filtrate, a small portion of its central nervous system will originate the disease in a fresh animal. The infective agent is thus placed among the filterable viruses. The resistance of the virus to external agents varies. Thus tissue of the nervous system containing it may be virulent even after the onset of putrefaction ; it can resist the prolonged application of a temperature of from $-10°$ to $-20°$ C., but, on the other

hand, it is rendered non-virulent by one hour's exposure at 50° C. Again, its potency probably varies in nature according to the source. Thus, while the death-rate among persons bitten by rabid dogs is about 16 per cent., the corresponding death-rate after the bites of wolves is 80 per cent. Here, however, it must be kept in view that, as the wolf is naturally the more savage animal, the number and extent of the bites, *i.e.* the number of channels of entrance of the virus into the body and the total dose, are greater than in the case of persons bitten by dogs. As we shall see, alterations in the potency of the virus can certainly be effected by artificial means, such as drying, heating, and applying chemical agents. Various



(*a*) (*b*)

FIG. 172.—Negri Bodies in Hippocompus of rabid dog. Section stained by eosin and methylene-blue. ×1000. (*a*) Two bodies within a nerve cell; (*b*) a body showing internal structure.

attempts have been made to obtain cultures of the rabies virus, but convincing results have not been obtained.

Negri bodies.—In 1903, Negri described certain bodies as occurring in the nervous system in animals dying of rabies to which much attention has since been devoted, and regarding the significance of which opinion is still divided. Negri's observations have been fully confirmed, and the occurrence of these bodies may be regarded as specific.

The Negri bodies (Plate IV., Fig. 17 [1]; Fig. 172) can be readily found in the affected nervous system by making smear preparations or sections, as described below. They vary much in size, measuring 0·5–25 μ; in the dog as a rule they are 4–10 μ. They are round, oval, or somewhat angular in outline. They are found in the protoplasm of the nerve cells and of their pro-

[1] For the material from which this preparation was made we are indebted to Lt.-Col. W. F. Harvey, I.M.S.

cesses. When examined in unstained preparations, they are seen to have a sharply defined outline. Their exact staining reactions vary with the method used, but they tend to be eosinophile. They appear not to contain constituents which give the microchemical reactions of chromatin.

For the finer differentiation of the internal structure, Negri employed Giemsa's stain. With this stain and under high magnification the groundwork of the body is a pale blue; in it there appear certain round or oval formations, single or multiple, of varying size, stained pink and of homogeneous appearance (*grosse Innenformationen*). In addition, both inside these larger formations and in the general substance of the body are smaller red or violet-red granules, occurring singly or in clumps (*kleine Innenformationen*). Though the description given applies to Giemsa preparations, any eosin-methylene-blue stain is suitable for their demonstration.

The Negri bodies have been found in practically 98 per cent. of cases of street rabies in dogs examined by many observers in different parts of the world. They are also found in natural rabies in other animals, and are usually present in human cases. Numerous control observations on other toxic conditions of the nervous system, especially where these are characterised by spasms, have been made, and the consensus of opinion is that the presence of Negri bodies is a specific appearance in nerve cells and justifies a positive diagnosis of rabies. The bodies occur in all parts of the nervous system, but are specially abundant in the cells of the hippocampus major and in the Purkinje cells of the cerebellum. It is in the former situation that they are generally looked for. They are apparently not so readily found, and at any rate the larger forms may be altogether absent, in animals dying from inoculation with the exalted fixed virus (*vide infra*). Hitherto they have not been certainly found in the salivary glands or saliva of a rabid animal. In infections produced by the fixed virus very small acidophile granules have been observed in large numbers in the nerve cells (Manouélian).

There has been great difference of opinion as to the true nature of these inclusion bodies. Negri himself regarded them as protozoa. This view is based upon their relatively constant and peculiar structure which, according to such authorities as Golgi, does not correspond to any cellular degeneration. And the fact that the nerve cells in which they are present may show no evidence of degeneration, is also noteworthy. Against their protozoal nature has been urged their absence from the brains of animals dying from fixed virus, their non-discovery in the infected saliva, and the fact that the virus can pass through

a filter. Further, in appearance they do not correspond to any known type of protozoal organism. These objections have been met with the argument that the smaller internal formations may be the infective agent in its essential form, and a modification of this view is that the Negri body is the result of a cellular reaction to the invasion by these minute forms. Manouélian and Viala have regarded the Negri body as an aggregate of minute elementary corpuscles, representing the causal organism, which they have named *Encephalitozoon rabiei*.

The Prophylactic Treatment of Rabies.—Until the publication of Pasteur's researches in 1885, the only means adopted to prevent the development of rabies in a person bitten by a rabid animal had consisted in the cauterisation of the wound. Such a procedure was undoubtedly not without effect. It has been shown that cauterisation within five minutes of the bite prevents the disease from developing, and that if done within half an hour it saves a proportion of the cases. After this time, cauterisation only lengthens the period of incubation ; but, as will be seen, this is an extremely important effect.

The work of Pasteur, however, revolutionised the whole treatment of wounds inflicted by rabid animals. Pasteur started with the idea that, since the period of incubation in the case of animals infected subdurally from the nervous systems of mad dogs is constant in the dog, the virus has been from time immemorial of constant strength. Such a virus, of what might be called natural strength, is usually referred to in his works as the virus of *la rage des rues*,[1] in the writings of German authors as the virus of *die Strasswuth*. Pasteur found that when the virus of *la rage des rues* was passed by subdural inoculation through a series of rabbits or guinea-pigs, its virulence was exalted for these animals till a constant strength (the *virus fixe*) was attained—constancy of strength being indicated by the unvarying occurrence of paresis on the sixth day. It may be noted here that the fixed virus while of high virulence on subdural injection is usually avirulent on subcutaneous inoculation. Pasteur also elaborated a method by which the exalted virus contained in the spinal cords of rabbits could be attenuated. This was done by drying the cords in air over caustic potash (to absorb the moisture), the diminution of virulence being

[1] While Pasteur's original statements regarding the constancy of the virulence of the street-virus were probably accurate for the street dogs of Paris, it has been found that if the general virulence of virus derived from animals in nature be studied, considerable variation occurs. It is now usual to apply the term street-virus to any virus derived from an animal becoming rabid under natural conditions of infection.

proportional to the length of time during which the cords were kept. Accordingly, by taking a series of such spinal cords kept for various periods of time, he was supplied with vaccines of different virulence. He found that, commencing with the subcutaneous injection of attenuated virus, and following this up with the injection of the stronger varieties, he could immunise dogs against subdural infection with a virus which, under ordinary conditions, would certainly have caused a fatal result. Pasteur then applied himself to find whether the comparatively long period of incubation in man could be taken advantage of to "vaccinate" him against the disease and thus to produce active immunity before its gravest manifestation developed.

In the earliest case treated the first injection was emulsion of rabbit's cord dried for fourteen days, and this was followed by injections of cord less attenuated. Treatment was continued over nine days, the last injection being one of emulsion of cord dried for only one day, *i.e.* containing approximately *virus fixe*. The patient never manifested the slightest symptom of hydrophobia. Other similarly favourable results followed ; and this prophylactic treatment of the disease quickly gained the confidence of the scientific world. It should be noted that apart from the attenuation produced by desiccation, the rabies virus after repeated passage in rabbits becomes less virulent to man.

A modification in the method was introduced later in serious cases, such as multiple bites from wolves, extensive bites about the head, and cases which came under treatment at a late period of the incubation stage. In these the immunisation was expedited, an emulsion of cords of one day's desiccation being injected on the third day of treatment.

The procedure now used in the Pasteur Institute of Paris is substantially the same as the original method, though certain modifications have been introduced as regards the preparation of the vaccine, its dosage and virulence (see Viala). Methods, however, vary in different Pasteur institutes. The most important modifications which have within recent times taken place are (1) the substitution by Högyes of increasing concentrations of a fairly fresh virulent rabbit's cord for emulsions of cords subjected to decreasing periods of drying, and (2) the use of material prepared from the brain and medulla of rabbits infected with fixed virus—the virus having been inactivated and preserved with carbolic acid (Semple) or ether (Alivisatos, Hempt). Equally good results apparently are obtained by these methods (McKendrick), but it is difficult from the available statistics to draw any exact comparisons among the different

forms of vaccine as regards their relative immunising value. Semple's method is now widely used. It is stated that in cases treated by the modified methods, certain symptoms sometimes following the original treatment, the gravest of which may be the occurrence of paralysis (" neuroparalytic accidents "), are not so frequently observed. This, according to Harvey and McKendrick, may be due to the fact that a smaller amount of nerve tissue is injected under the Högyes system, and also that phenol destroys the toxic property of nerve tissue. Stuart and Krikorian, however, have recently recorded such neuroparalytic accidents following the administration of carbolised vaccine. They advance the theory that the nerve substance of antirabic vaccines contains a cytotoxin to which certain persons are peculiarly susceptible.

The success of the treatment has been very marked. The statistics of the cases treated in Paris are published annually in the *Annales de l'Institut Pasteur*. Besides the Institute in Paris, similar institutions have been established in various parts of the world. At Kasauli, India, during the period 1900–25, 98,420 cases were treated, with a mortality of 1·23 per cent. The collected statistics of a large number of institutions in various countries have shown a mortality of about 1 per cent., and of late years the results have been even more favourable. It is difficult to determine the ordinary mortality in untreated persons, but this has been estimated at 5 to 10 per cent. or even higher. It has been alleged that many people are treated who have been bitten by dogs that were not rabid. This, however, is not more true of the cases treated by Pasteur's method than it was of those on which the ordinary mortality of untreated cases was estimated Following Pasteur, care is taken in making up the statistics to distinguish three classes of cases. Class A includes only persons bitten by dogs proved to have had rabies, by inoculation in healthy animals of parts of the central nervous system of the diseased animal or by the demonstration of Negri bodies. Class B includes those bitten by dogs that a competent veterinary surgeon has pronounced to be rabid. Class C includes all other cases. At Paris during 1895, 122 cases belonging to Class A were treated, with no deaths ; 940 belonging to Class B, with two deaths ; and 449 belonging to Class C, with no deaths. In 1931, 531 persons in all were vaccinated with no deaths (Viala).

Extensive experimental evidence bearing on the efficacy of prophylactic immunisation is afforded by the work of Harvey and Acton ; using monkeys, they obtained 6 survivals out of 34 animals immunised with virus before inoculation, whereas of an equal number which either were untreated or had received normal brain suspension before inoculation only 1 survived.

Antirabies Serum.—Valli showed that immunity against rabies could be conferred by administering through the stomach progressively increasing doses of the rabies virus. Following up this observation, Tizzion and Centanni attenuated rabies virus by

submitting it to peptic digestion, and immunised animals by injecting gradually increasing strengths of such virus. This method is usually referred to as the Italian method of immunisation. The latter workers described both prophylactic and curative effects obtained experimentally with their antiserum. Marie obtained a similar serum by subcutaneous injection of sheep with *virus fixe*, which prevents the occurrence of the disease when a mixture with the virus is injected into susceptible animals. This serum has been used to supplement the ordinary Pasteur treatment.

Diagnosis.—*Demonstration of Negri bodies.*—The animal should be killed and the brain removed after reflecting the scalp and cutting through the calvarium with a sharp chisel. The brain is placed with the vertex uppermost, and the upper parts of one hemisphere are removed in thin horizontal slices till the anterior part of the lateral ventricle is reached. The roof of the ventricle is then cut away with a probe-pointed bistoury; the hippocampus is recognised as the laterally arched ridge which forms the floor of the ventricle. This may be transversely incised and thin slices removed for the making of smears and sections. A portion should also be taken from the cortical grey matter of the cerebrum and from the cerebellum. Smear preparations may be made as follows: a thin portion of nervous tissue is placed on a glass slide towards one end, on this a cover-glass is placed, and then, gentle pressure being exerted, a smear is made by sliding the cover-glass towards the other end of the slide. These are fixed while still wet with methyl alcohol (one minute) For staining, any methylene-blue-eosin combination may be used, but Mann's stain or Ford's modification as described on page 724, is recommended.

Animal inoculation —In addition to microscopic examination, a small piece of the medulla or cord of the suspected animal should be taken, with aseptic precautions, rubbed up in a little sterile 0·85 per cent. sodium chloride solution, and injected by means of a syringe beneath the dura mater of a rabbit (p. 162). Material contaminated with bacteria should be inoculated intramuscularly In rabies in the rabbit, symptoms of paresis usually occur in from six to twenty-three days, and death in fifteen to twenty-five days. In this way conclusive evidence is afforded of the presence of rabies infection in the suspected animal When the material for inoculation has to be sent any distance, it is best to pack the head of the animal in ice. The virulence of organs is preserved, however, by placing them in glycerol.

It has also been recommended that the suspected animal should be kept till it dies and the brain then examined as above, since positive results are more likely to be obtained under these conditions.

Pseudo-rabies (Mad Itch)

This is an acute infective disease occurring in various animals, *e.g.* cattle, in Europe and America and characterised by intense itching. Though named pseudo-rabies it bears only a superficial resemblance to true rabies. The disease has been studied by a number of workers (Aujeszky; Remlinger and Bailly Shope; Hurst; and others). The condition is highly fatal in some animals and runs a rapid course after a short incubation period, paralysis occurring as a terminal result. The manifestations, however, may

be variable. The infection is not transmissible to man. Histological changes are observed in the spinal cord and spinal ganglia ; these consist mainly of cellular degeneration. The disease has been proved to be due to a filterable virus and can be reproduced by experimental inoculation in a variety of animals including the rabbit. The virus has been distinguished from that of rabies. It is present in the nervous system, internal organs and in the blood. By filtration through graded collodion membranes the virus particles have been estimated to be 0·1–0·15 μ in diameter. The virus has been cultivated in tissue cultures and chick-embryo medium. Immunisation of animals yields an immune serum which inactivates the virus.

CHAPTER XXV

FILTERABLE VIRUSES (*continued*)

Epidemic Poliomyelitis

While the occurrence of "infantile paralysis" (Heine-Medin disease) of sudden onset, and affecting especially one or more limbs, has been known since the earliest times, it is only coincident with the modern developments of neurology that the most prevalent type has been recognised to be associated with inflammatory changes which are specially concentrated in the anterior cornua of the spinal cord. Though the disease chiefly attacks children, older subjects are also affected, and in some epidemics the infection of adults is a prominent feature. The disease is usually sporadic in its incidence, and, as has long been known, in temperate climates it is of most frequent occurrence during the warmer months of the year. It also occurs in an epidemic form. Such outbreaks have been well known in Norway and Sweden during the last century, but in other countries similar epidemics, limited or extensive, have come under notice. Thus in New York in the summer of 1907 an outbreak of probably over 2000 cases occurred, 762 of which were carefully investigated by a special Commission, and it is from their work that much of our present knowledge of the disease has been derived, and many facts regarding its infective nature have been definitely established. An even more serious epidemic took place in New York in 1916.

Clinically, the onset of the condition is marked by more or less pronounced fever, often accompanied by sore throat and followed after a few days by signs of paresis and paralysis; as a rule, in only a relatively small proportion of cases does death result, though there is a great variation in the mortality in different outbreaks. When recovery occurs, many of the paralytic symptoms may pass off, but generally there remains evidence of definite permanent injury to the motor functions of the nervous system. Pathologically, the early lesions consist in a local or general leptomeningitis with pronounced exudation of polymorphonuclear leucocytes and lymphocytes into the perivascular spaces, the existence of which is reflected in the appearance of such cells in moderate numbers

in the cerebro-spinal fluid. In the later stages the cells present are mainly the lymphocytes. In the cord the inflammatory condition is usually marked in the arterioles of the anterior commissure, especially in the cervical and lumbar regions, and thence passes into the anterior cornua along the vessels, which show intense hyperæmia with perivascular cell proliferation, and which may become thrombosed or rupture. The nerve cells may die and become the prey of neuronophages, and secondary local and systemic degenerations may follow. Such a pathological picture, however, is not confined to the grey matter nor indeed to the cord, as similar changes have been observed in the brain. The recognition of this has widened the whole conception of the disease, and various clinical types besides the classic anterior poliomyelitis are now recognised to exist. These depend partly on variations in the severity of the condition, partly on the disease being concentrated in a particular part of the nervous system. These less common types probably include many cases described as the acute ascending paralysis of Landry, acute bulbar paralysis, cases characterised by acute meningitis or encephalitis, cases of rapidly developing ataxia, and even cases simulating neuritis. It has been supposed that the death of nerve cells is a consequence of interference with their nutrition following the vascular lesions described above. But according to Hurst and others, if monkeys infected with a highly virulent virus are examined at a suitably early stage of the disease, severe damage of the nerve cells can be demonstrated without any associated vascular lesions or cellular exudate. Accordingly this is to be regarded as the initial lesion.

Experimental Transmission.—The infectivity of the disease was established by the work of Landsteiner and Popper, who in 1909 in Vienna succeeded in producing the disease in a monkey by the intraperitoneal injection of an emulsion of the spinal cord of a child who had succumbed on the fourth day of illness. Similar observations were made in the same year by Flexner in New York, who found that if for intraperitoneal injection intracerebral inoculation was substituted, positive results were more uniformly produced, and the brain and cord of the infected animals were infective for other monkeys, the incubation period being from four to thirty-three days. Hitherto the monkey is the only animal to which the disease has certainly been communicated—both the anthropoid apes and the lower monkeys are susceptible, and the conditions resulting from inoculation are clinically and pathologically identical with those observed in man. Although rabbits may occasionally develop the disease after experimental inoculation, this is exceptional.

In infecting monkeys from a human case it is advisable to commence with the use of an unfiltered emulsion of the central nervous system, for filtered emulsions possess much less virulence; but after a few *passages* through monkeys it is found that filtration has little effect in diminishing the number of successful

inoculations, the virus being now so potent that 0·001 to 0·01 c.c. of an emulsion of material from the central nervous system (p. 756) in distilled water will originate the disease when injected into the brain. The disease can be originated by subdural and intracerebral injection, and also by introduction into the sheath of such a nerve as the sciatic. When the sheath of a nerve is injected, the paralytic symptoms usually first appear in relation to that part of the cord from which the nerve emerges. Infection can also readily be produced by scarifying the mucous membrane of the nose and rubbing the virus into it, or even by simply injecting it into the nasal cavities. The intraperitoneal, intrathecal, cutaneous and subcutaneous routes can also be employed, but to cause the disease by intravenous injection enormous doses must be administered. It is an important fact that when different strains are compared, some are more virulent on inoculation by the intracerebral route than by the nasal or cutaneous, whereas with others the opposite is the case.

Properties of the Virus.—The discovery was made independently by Flexner and Lewis, and by Landsteiner and Levaditi, that it could pass through an earthenware filter (*e.g.* Berkefeld N or V). The deduction from this was that the causal organism must be very small, and Flexner and Noguchi succeeded in cultivating minute bodies which they believed to be the infective agent. Small portions of the central nervous system—preferably the brain—removed *post mortem*, were inserted in a medium composed of naturally sterile ascitic fluid containing a fragment of sterile fresh rabbit kidney, and the cultures incubated at 37° C. under anaerobic conditions. About the fifth day faint opalescence appeared, and the fluid was found, when treated with the Giemsa stain, to contain minute bluish or violet globoid bodies, about 0·2 μ in diameter, in pairs, chains, or, less commonly, in groups. Towards Gram's stain their behaviour was variable. Similar cultures were got from the infective filtrates. Further, the organism could adapt itself to other media and could be maintained in subculture. By inoculating monkeys with these cultures, under precautions which excluded the possibility of infection being derived from the brain matter originally used, poliomyelitis was set up in the animals, and the organism was recovered from their brains. These "globoid bodies" were also demonstrated by Giemsa's stain, in the brain in both the natural and experimental disease. Recent observations by Theiler and by Elford and others have indicated that the virus is much smaller (8–17 mμ). Successful transmission of the disease by cultures has also been recorded

by Gildemeister, but most attempts have failed. Gildemeister's medium consisted of Tyrode solution, 10 per cent. monkey serum and minced brain of a ten to twelve days' chick embryo ; subcultures were made twice weekly and material of the eighteenth passage caused a fatal infection in a monkey.

Rosenow and Towne look on the "globoid bodies" as specially small forms of a rather large streptococcus which they have isolated from the brain in both the natural and experimental disease—which can grow under aerobic conditions and can produce poliomyelitis, not only in monkeys but also in rabbits These organisms have been found in certain cases by other observers, who, however, deny that the disease conditions they produce are to be classified with poliomyelitis. The streptococci are most probably occasional secondary invaders

The virus withstands glycerolation for years, and can be kept frozen at $-2°$ to $-4°$ C. without being affected. It also withstands from 1 to $1\frac{1}{2}$ per cent. phenol for at least five days ; it is, however, killed by an exposure at 50° to 55° C. for one-half to one hour.

Pathology of the Disease.—By means of the inoculation method the distribution of the virus in the natural and experimental disease has been determined and has been found to be similar in both cases. The virus is markedly neurotropic, and is highly concentrated in the brain and spinal cord. It also occurs in the intervertebral ganglia, the Gasserian ganglion, and in the abdominal sympathetic ganglia. It may be found in the lymphatic glands, especially the tonsil and those of the mesentery, and it has been demonstrated in the nasal mucous membrane. It is absent from the other solid organs and the blood.

Flexner's view of the pathology of the disease is that infection takes place through the nasal mucous membrane, a catarrh of the buccal and nasal cavities being often the first sign of the disease. In monkeys in which intracerebral inoculation has been practised the virus is eliminated into the nose, and the nasal mucus has been found to be infective in human cases. When an individual is infected by the inhalation of such mucus, it is probable that the virus gains access to the brain along the olfactory nerve route ; this view rests on the observation that when monkeys are inoculated by painting the infective material on the nasal mucosa, the olfactory lobe becomes infected before other parts of the brain. This fact, as well as the size of the dose required to produce infection by intravenous injection, militates against the possibility of the virus being carried to the central nervous system by means of the

blood under natural conditions. There is evidence from experimental intravenous injections that the choroid plexus, which is the source of the cerebro-spinal fluid, prevents (so long as it is uninjured, *e.g.* by inflammation) the passage of virus into the subarachnoid space. It is likewise possible that in natural infection the virus may pass into the mesenteric nodes and thence be absorbed by the lymphatics of the spinal nerves. All the facts point to the importance of the part played by the nerves in the passage of the causal agent to the central nervous system. This is confirmed by the series of cases reported by Leake in the United States, where the disease followed inoculation with a living virus given for purposes of immunisation. In every instance the lesion first developed in the spinal cord at the level corresponding to the limb into which the material had been injected. The exact route by which the virus is transported in the nerves—whether by lymphatics, which seems unlikely on anatomical grounds, or in the axis cylinders—remains unsettled. While the virus may be said to be neurotropic, the term must be used in the sense that all the elements of the nervous system—pia-arachnoid, glia, interstitial blood vessels as well as parenchymatous cells—show a special susceptibility. The pathological anatomy in these structures has been described above. Intranuclear acidophile inclusion bodies have been described in the nerve cells in human cases by Hurst. In monkeys they are seldom seen, apparently owing to the rapid occurrence of necrosis of affected cells.

These observations have furnished important indications of the method by which infection takes place, and by which both the sporadic cases and the epidemic outbreaks occur. It has been found that in monkeys recovered from the disease, the nasal mucosa remains infective for many months after the virus has disappeared from the central nervous system, and it has been established that in man there are chronic carriers such as exist in other diseases. As in other conditions, the carrier may not himself suffer from the effects of the infective agent which he carries. Further, the occurrence of abortive cases may constitute a means by which infection is maintained in a community. Such abortive cases are probably fairly common during epidemics. In connection with this aspect of the subject, Amoss and Taylor have made the interesting observation that in some individuals the normal nasal secretion possesses a certain power of neutralising the poliomyelitic virus. Finally, it is to be noted that there is a periodicity in the incidence of poliomyelitis in an epidemic form. As bearing on the explana-

tion of this, Flexner, Clark and Amoss record the case of one strain of the virus the virulence of which in monkeys was at first low, and then rose to a maximum which was maintained for three years; this phase was succeeded by a decrease in infectivity in a few months, without apparent cause. It is obvious that this fact is not only of importance in relation to poliomyelitis, but is suggestive as bearing on the periodicity of other epidemic diseases.

Rosenau has put forward observations pointing to a blood-sucking fly, *Stomoxys calcitrans*, being capable of transferring the disease in monkeys As the worst epidemics of poliomyelitis occur in summer, the possibility of an insect carrier has thus been entertained. The absence of the virus from the blood in man, and the difficulty of originating the disease by intravenous injection, are facts militating against such a theory.

Immunity Phenomena.—Though no cases are recorded of a second attack of poliomyelitis in man, our knowledge regarding immunity is mainly derived from animal experimentation. Monkeys which have passed through an attack of the disease are insusceptible to fresh inoculation; but definite disease manifestations are apparently essential to the establishment of immunity, as animals which have at first yielded negative results are usually susceptible to a second inoculation. Both in man and in the monkey the serum of a recovered case contains substances capable of neutralising the virus, for if such serum be mixed with virus and incubated for a time at 37° C. the mixture becomes inoperative on intracerebral injection into monkeys. The antibodies persist in the serum in man for many years after an acute attack, and they possess this further significance, that they may be found in the so-called abortive cases where a transient illness with little or no involvement of the nervous system occurs. The only definite evidence that such a condition is due to the virus of poliomyelitis lies in the fact that subsequently the serum has the capacity of neutralising the virus. According to Jungeblut the virus-neutralising property is more frequent and more abundant in normal individuals of blood-group B than in those of the other groups. Not only has the immune serum neutralising properties *in vitro*, but it has been shown experimentally to have a certain effect *in vivo* when introduced intrathecally into monkeys previous to intravenous inoculation. Trask and his co-workers on comparing the immunity produced by a number of strains, have found differences. Strains isolated from the same epidemic appear to be more closely related immunologically than those from different epidemics.

The serum of recently recovered human cases has been injected for therapeutic purposes into patients suffering from poliomyelitis (especially during the first forty-eight hours) in amounts of 35 to 120 c.c., administered both intrathecally and intravenously. Early reports suggested that paralysis was arrested by the treatment; but extended experience in America and Europe has not confirmed its value. Flexner and Stewart have also recommended a subcutaneous injection of convalescent serum as a prophylactic measure, the dose being 10 to 20 c.c. according to age, which may be repeated four to six weeks later if required. It has been stated by Kraus that if virus which has been killed, *e.g.* by phenol, is injected into monkeys they develop resistance, but the general experience is that dead virus fails to immunise. Stewart and Rhoads have found that in monkeys a considerable degree of protection against intracerebral inoculation may be developed by a series of intradermal injections of the living active virus. Accordingly the use of virus attenuated by various means, such as treatment with formalin or sodium ricinoleate, has been advocated. These preparations are unsafe, however, as Leake has reported twelve cases in which an attack of poliomyelitis followed their use, paralysis occurring usually about six to fourteen days after the initial injection. The experiments of Fairbrother and Morgan indicate that it may be possible to obtain antiviral serum from actively immunised horses.

Diagnosis.—The fact that poliomyelitis appears under a variety of clinical types makes the diagnosis difficult in many cases, particularly of mild illness. This is specially true of the meningitic type, which may be difficult to distinguish from epidemic cerebro-spinal meningitis, especially as the characters of lumbar puncture fluid in the two diseases are very similar, and, as is known, it may often be difficult to isolate the meningococcus where it is actually the causal organism.

Methods.—The inoculation of a monkey constitutes the only certain means of diagnosis in a doubtful case of poliomyelitis. A piece of spinal cord removed with all aseptic precaution from a fatal case is ground up with saline (the nervous tissue forming 10 per cent. of the whole). The product is allowed to settle and 1 to 2 c.c. of the supernatant fluid is injected intracerebrally. When symptoms develop the animal should be killed and its nervous tissue used to inoculate another monkey; otherwise recovery of the first animal tends to occur and the virus is lost. Portions of the central nervous system may be placed in 33 per cent. glycerol for transmission to a laboratory. In certain cases information might be obtained by injecting material from swabs of sterile wool allowed to remain in the nasal passages, in order that the mucus may be absorbed.

Portions of tissue removed from the tonsils might also be useful; in each case the material may again be immersed in small quantities of glycerol, or advantage may be taken of the fact that the virus can survive exposure to 1 per cent. phenol for several days.

Cases have occurred where the diagnosis lay between poliomyelitis and the paralytic type of rabies, and in the present stage of knowledge the susceptibility of the rabbit to the latter disease would constitute the chief means by which the diagnosis could be arrived at

Epidemic Encephalitis

A number of outbreaks of encephalitis have now been recorded in various parts of the world. The most extensive was that to which the name of Encephalitis lethargica was applied. But in other epidemics differences have been observed in the susceptibility of animals to inoculation with the disease, as well as in the clinical features and the anatomical changes. Therefore it appears that we have to deal with a group of infections. The subject is further complicated by the natural occurrence of similar diseases in animals. Also, certain viruses which do not ordinarily produce encephalitis in man, may do so in animals under suitable conditions as to virulence and route of inoculation.

Encephalitis lethargica

During the spring and summer of 1918 a number of cases of encephalitis occurred in Britain which were characterised clinically by lethargy and drowsiness, often passing into coma, with moderate or no rise of temperature. A great variety of nervous symptoms were recorded—headache, epileptic fits, spastic phenomena, ascending paralysis, etc.—but the most common and striking feature was the existence of irritative and paralytic affections of the muscles of the eyelids and eyeballs. The mortality was high. In fatal cases the chief post-mortem changes were small sub-pial hæmorrhages and hæmorrhages into the grey and white matter of the brain. There was sometimes marked subdural œdema. Meningitis was not a marked feature, and when it occurred, was patchy; the cerebro-spinal fluid was usually clear, there being at first a slight lymphocytosis which disappeared later. Microscopically, the hæmorrhages appeared to be of venous origin; there was intense capillary congestion and patchy perivascular round-cell infiltration, as well as focal collections of these cells in the substance of the brain. The lesions affected especially the pons, medulla, and mid-brain; degenerative changes in the oculo-motor centres were recorded. The occurrence of ophthalmoplegia suggested

that the condition was botulism, but the symptoms of the two diseases did not otherwise correspond. No association with the taking of particular articles of food was traceable, and no evidence was obtained that *B. botulinus* was responsible for the condition. It was also suggested that the condition was poliomyelitis of an aberrant type, but the findings differed in certain respects from those of the cerebral cases which have been observed during epidemics of poliomyelitis, and, further, there was no evidence of a concurrent prevalence in Britain of ordinary poliomyelitis. It is noteworthy that in encephalitis the cellular infiltrations in the brain have been found to consist mainly of lymphocytes, while neutrophile polymorphs are rare, whereas the latter cells are numerous in the corresponding lesions of poliomyelitis. The condition was not confined to Britain, an outbreak having been recorded in Austria during 1917, and in France in 1918. In the British cases the bacteriological findings were as a rule negative. In support of the separate identity of poliomyelitis and encephalitis is the fact that the serum from recovered cases of the latter disease is devoid of anti-infective action on the virus of poliomyelitis.

Since encephalitis lethargica was first recognised as an epidemic disease in this country the condition has been specially prevalent in urban districts. The disease has shown an increased incidence during the first quarter of the year, which contrasts with the seasonal prevalence of epidemic poliomyelitis. The infective nature of the condition has been generally accepted, and attention has been called to the occurrence of mild or ambulatory cases. This has raised the question of infection being spread by "carriers," as in the case of epidemic poliomyelitis. It has been suggested that the infection atrium is the upper respiratory tract. A condition of so-called "epidemic hiccup" which has been reported in recent years has also been regarded as a manifestation of epidemic encephalitis.

Extensive investigations have been made into the nature of the infection, but so far without conclusive results. As a result of experiments in which encephalitis has been reproduced in animals by intracerebral inoculation with material from the human disease (*vide infra*), the virus has been regarded as one of the filter-passers. Loewe and Strauss have reported the cultivation from the disease of minute globoid bodies similar to those described in poliomyelitis (*vide* p. 752), and Rosenow has claimed that the condition is due to a type of streptococcus. These observations have not been confirmed. Various workers have observed minute granular structures (Da Fano's "minute

bodies ") situated intracellularly in the lesions, similar to those described in experimental herpetic encephalitis (*vide* p. 765). Though it has been suggested by some that these cell inclusions represent the virus, others, *e.g.* Cowdry and Nicholson, hold that the structures in question are merely cell-derivatives. In the interpretation of the results of experimental transmission of the disease to laboratory animals, a serious fallacy has been brought to light, namely, the occurrence of spontaneous encephalitis in certain of these animals, *e.g.* rabbits and monkeys, from which they may recover, though the histological lesions, in the form of round-cell infiltrations, may persist for some time.

In 1920 Levaditi and Harvier reported that they had transmitted experimentally the virus of encephalitis lethargica and demonstrated its pathogenicity for certain laboratory animals, *e.g.* rabbits, the lesions being the same as in the human subject, and similar results have been reported by McIntosh and others. They claimed that it was filterable, and, like certain other viruses of this nature, could be preserved in glycerol, and concluded that it was conveyed to the central nervous system by cranial, ocular, and peripheral nerve routes, but was not pathogenic if injected subcutaneously, intravenously, or intraperitoneally, or if introduced into the trachea or stomach. McIntosh and Turnbull in the same year claimed to have reproduced the disease in a *Patas* monkey by combined subdural and intraperitoneal inoculation of filtered emulsion of the cervical cord, pons, and basal nuclei from a fatal human case. The animal developed convulsive attacks and ultimately became lethargic and died on the fifty-sixth day. The microscopic lesions in the brain resembled those in the human disease. From the monkey McIntosh transmitted the infection to other monkeys and also to rabbits—an acutely fatal disease, however, developing only in the latter. It may be said that the successful transmissions to animals which have been reported are few in proportion to the failures. Levaditi and his co-workers, and others, have shown that the viruses isolated by them from cases of encephalitis produce a characteristic train of symptoms which are identical with those produced by a neurotropic strain of the herpes virus (*vide* p. 763). Further facts of importance are that these viruses also produce herpetic keratitis in rabbits, and that, as shown by Levaditi in cross immunity experiments, the virus of herpes febrilis and his supposed encephalitis virus are identical. It is worthy of note that Flexner and Amoss produced encephalitis in rabbits by intracerebral injection of the cerebrospinal fluid from a case of neuro-syphilis which showed no

evidence of encephalitis. It seems likely that the herpes virus, undoubtedly widely prevalent, may become generally distributed in the tissues and fluids of the body. Thus, if all the facts are taken into consideration, the possibility must be considered that the virus recovered from cases of encephalitis lethargica is merely the herpes virus. On the other hand, the view that encephalitis and herpes in the human subject are merely manifestations of the action of the same virus can hardly be accepted on the evidence at present available. According to McIntosh and Turnbull, in the experimental disease which they produced, the lesions consisted of a non-purulent cellular encephalitis with infiltrations of the perivascular sheaths consisting mainly of lymphocytes and plasma cells; further, inoculation of the cornea did not lead to keratitis. The intranuclear inclusion bodies found in the nerve cells in herpes encephalitis (p. 765) are absent in encephalitis lethargica. If we exclude as inconclusive the experiments in which the sole evidence of disease in the inoculated animals has been the finding of microscopic lesions in the brain, then of the many attempts to transmit encephalitis with material from the human subject very few have been successful. Attempts to cultivate these viruses by various methods have proved unsuccessful.

St. Louis Encephalitis

An extensive epidemic of encephalitis occurred in St. Louis, U.S.A., in the autumn of 1933, the highest incidence and mortality being in middle-aged and old people. The lesions resembled those of encephalitis lethargica, but at the beginning of the epidemic especially, were more severe. *Macacus rhesus* monkeys were infected by combined intracerebral and intraperitoneal inoculation with emulsions of brain from cases, but were not highly susceptible, and the infection tended to die out after several passages. The lesions in monkeys resembled those in man. Mice proved susceptible to intracerebral or intranasal inoculation, and after an incubation period developed acute encephalitis which was fatal in a few days. Passage through mice could be continued indefinitely. The serum of convalescent human cases or monkeys possesses the power of neutralising the virus, as tested by inoculating mice with the mixture. By such protection tests neutralising bodies were demonstrated in 30 per cent. of a series of sera from inhabitants of a number of American cities; on the other hand, only 9·4 per cent. of 113 normal controls without known exposure to the

disease possessed antibodies (Wooley and Armstrong). Thus a wide distribution of the virus was indicated. It was shown also by protection tests that the disease is immunologically distinct from poliomyelitis, encephalitis lethargica, and forms of post-infective encephalitis. The virus measures 20 to 30 mμ ; it can be preserved by freezing, but rapidly becomes inactive in 50 per cent. glycerol. It appears to be distinct from other filterable viruses hitherto described.

Japanese Encephalitis.—Large outbreaks of encephalitis with high mortality tend to occur in Japan in the late summer. Taniguchi and others have recovered by intratesticular inoculation of rabbits with the cerebro-spinal fluid of a case, a filterable virus which causes encephalitis in rabbits and monkeys on subdural inoculation. Elementary bodies have been demonstrated in the endothelial cells of the cornea of the rabbit after inoculation of the anterior chamber with the virus. It was highly resistant to glycerol. This virus was virulent for mice and rats on intradermal inoculation and in this respect seemed to differ from other viruses described in encephalitis, also its separate identity from virus III and *Encephalitozoon cuniculi* (*vide infra*) was determined ; but it is left undecided as to whether it is distinct from that of herpes. Cross immunity tests on the skin of rabbits previously immunised by intratesticular inoculation also indicated a distinction from the viruses of vaccinia, varicella or herpes.

Kasahara and others have also isolated from such patients viruses which apparently differ from the above.

Australian Encephalitis (X-Disease).—A virus was recovered from cases in a highly fatal epidemic in New South Wales (Cleland and Campbell) It was readily transmitted to monkeys, in which it caused brain lesions resembling those of louping-ill (Perdrau), destruction of the Purkinje cells being a striking feature.

Acute Disseminated Encephalo-myelitis.—Of late years attention has been drawn to cases of encephalitis ("post-vaccinial encephalitis") occurring particularly in Holland and Great Britain about a fortnight after vaccination, a number of which have proved fatal, the majority, however, recovering completely. This condition has been met with chiefly in older children vaccinated for the first time. In view of the fact that fatal meningo-encephalitis can be produced in monkeys and rabbits by intracerebral inoculation with vaccinia virus, the question has arisen as to whether the condition is causally related to vaccinia. A characteristic histological feature in the human cases which have lived for some time is the presence in the brain and cord of perivascular areas of demyelination (Turnbull and McIntosh ; Perdrau). These areas are situated most commonly in the subcortical white matter, in the thalamus, mid-brain and pons, and round the ependyma of the lateral ventricles ; the cord is affected especially around the margins, but the posterior columns tend to escape. A similar condition has been long known to occur sporadically after smallpox and measles, and is apparently also met with after other febrile illnesses and Pasteur's antirabic inoculation or even spontaneously. There is little to suggest that cases which have survived the acute stage develop into disseminated

sclerosis. In the post-vaccinial cases, the presence of vaccinia virus in the brain has only occasionally been demonstrated ; thus attempts to transmit the condition to animals by injecting suspensions of brain from fatal cases have generally failed. While the exact nature of the causal agent is undetermined, there is much evidence to support the view that it appears to be a virus or toxin acting on nervous tissues whose resistance has been depressed by various conditions, one of which is vaccination. However, the possibility cannot be entirely excluded that such encephalitis may be a manifestation of the action of the causal agent responsible for the primary disease. In post-vaccinial cases favourable results have been reported to follow an intravenous injection of 10 c c. of the serum of a person previously successfully vaccinated.

Encephalitis in Animals caused by Viruses.—Horses in Europe are affected by an encephalitis, called Borna disease from the locality where it was first described. The size of the virus bodies is 0·085–0·125 μ. In America there is an encephalitis of horses due apparently to a distinct virus. In the United States foxes suffer from encephalitis which may become epizootic at times. Louping-ill occurs in sheep on the borders of England and Scotland and in the Scottish Western Highlands, which was shown to be caused by a filterable virus. Mice are susceptible. The size of the virus bodies is 15–20 mμ. It is transmitted by the tick *Ixodes ricinus*. It is important that this disease is much aggravated by the simultaneous occurrence in the animals of another infection of unknown nature—" tick-borne fever." Cases of infection with the louping-ill virus in the human subject have been observed (Rivers and Schwentker).

Spontaneous Encephalitis of Rabbits.—This condition, first noted by Bull and subsequently studied by Oliver and by Twort and Archer, has been referred to above, and requires consideration in relation to the problem of epidemic encephalitis. It may be noted that the condition is usually associated with nephritis. It is readily transmitted both by cage infection and by experimental inoculation. The disease may be widespread. McCartney, working in America, found that about one-half of the stock rabbits examined by him showed encephalitis lesions, *e.g.* perivascular, meningeal, and sub-ependymal areas of round-cell infiltration and necrotic foci. It is of particular interest that Levaditi, Nicolau, and Schoen have described in this condition a micro-organism classified among the microsporidia and designated *Encephalitozoōn cuniculi*. This organism is found next to the subcortical areas of infiltration and occurs in groups of twenty to forty exceedingly small elongated or pear-shaped structures apparently enclosed in a cyst.

Lymphocytic Chorio-meningitis

A virus isolated by Armstrong and Lillie from a fatal case during the St. Louis outbreak of encephalitis was found to differ from that generally recovered. It produced in monkeys and mice an acute " aseptic " meningitis, the exudate in the meninges and choroid plexus being chiefly lymphocytic. Subsequently this virus was obtained from the cerebro-spinal fluid

of patients with mild meningeal or nervous symptoms in America and also in Great Britain (Findlay, Alcock and Stern). It is important that the same virus has been got from stocks of untouched mice. In man the disease appears to be mild as a rule; there may be an associated catarrh of the upper respiratory tract. The changes in the cerebro-spinal fluid are slight, the chief being a considerable increase of lymphocytes. The disease can be passed indefinitely through mice, which develop tremors five to twelve days after intracerebral inoculation and die in one or two days with spasms of the hind legs. The virus is present in the blood and various organs as well as the brain. Intranasal instillation of the virus produces the disease. Rats and guinea-pigs also develop a fatal infection. The serum of recovered individuals has viricidal properties.

Herpes Febrilis

Within recent years the etiology of herpes has been extensively studied, and it has been proved that the vesicular fluid of certain types of herpes (*e.g.* herpes febrilis, cornealis, genitalis) contains an infective agent or virus which appears to be filterable, although with difficulty. Grüter and Löwenstein first showed that the vesicular fluid of herpes febrilis was infective for rabbits when inoculated on the cornea, producing a herpetic keratitis similar to herpes cornealis in the human subject, the infection being transmissible in a series of animals. The virus was rendered inactive by exposure to a temperature of 56° C. for half an hour or by being kept outside the body at 37° C. for twenty-four hours. The experimental production of herpetic keratitis in rabbits by local inoculation with vesicular fluid of the above forms of herpes has been confirmed by subsequent workers. Also, it has been shown that material from the lesions in the rabbit, when inoculated into the skin or cornea in man, reproduces the typical herpetic vesicles. The herpes virus apparently possesses little capacity to attack the nervous system in the human subject, but its virulence for susceptible animals may be considerable. Thus, while the experimental keratitis in rabbits may ultimately clear up without any further effects, in a certain proportion of experiments, as first shown by Doerr and his co-workers, the virus attacks the central nervous system and produces meningo-encephalitis with death of the animal. The symptoms comprise pyrexia, muscular incoordination, salivation, retention of urine, teeth-gnashing, dashing movements, loss of equilibrium, and finally convulsions and paralysis. The changes

in the central nervous system are characterised by lymphocytic and leucocytic infiltrations, marked nerve-cell degeneration and proliferation of fixed tissue cells. This experimental condition has proved of great interest in view of its similarity to the changes in the brain in epidemic encephalitis and in animals inoculated with material from cases of this disease. A general herpetic infection in the rabbit has also been produced by intravenous injection of the conjunctival secretion of an animal with experimental keratitis (Doerr and Vöchting). It has been shown that the subdural inoculation in rabbits of material containing the virus either from the human subject or experimental keratitis produces a rapidly fatal encephalitis, and this condition is then transmissible in a series of animals by subdural inoculation with brain emulsion; further, corneal inoculation with brain emulsion produces a herpetic keratitis and in some cases encephalitis (Blanc and others). The herpetic keratitis of rabbits is also transmissible to guinea-pigs (Doerr and Vöchting and others). The vesicle fluid inoculated by scarification on other sites in the same individual may reproduce a herpetic condition. The virus has also been found in the blood and the spleen of experimentally infected animals; and in a human case the cerebro-spinal fluid has been shown to be infective for rabbits (Ravaut and Rabeau). (The virus of herpes genitalis is less virulent for the rabbit than that of herpes febrilis.)

The relationship of the herpes and epidemic encephalitis viruses has been suggested in view both of the experimental production of encephalitis by known herpes viruses and also of Levaditi and Harvier's observation that, by means of brain emulsion from rabbits infected with a supposed epidemic encephalitis virus, experimental keratitis could be produced followed by typical encephalitis. In fact, Levaditi, Harvier and Nicolau concluded that the herpes and encephalitis viruses were identical, the former being only a less virulent type of the latter. This has been considered above in regard to the etiology of epidemic encephalitis. Apparently the herpes virus may spread along the axis cylinders of motor and sensory nerves from the periphery to the central nervous system, and in the brain and cord the resulting lesions may be anatomically related to the nerves by which the virus has entered (Goodpasture). A rabbit which has recovered from experimental herpetic keratitis is immune to further inoculation. At first the immunity is most marked in the cornea of the same eye, but later the other cornea becomes insusceptible to inoculation, and this refractory state becomes intensified over a period of several months. After recovery

from corneal infection the animal is insusceptible also to intracerebral inoculation with herpes virus. According to Zinsser and Tang, the serum of recovered animals neutralises the virus when added *in vitro*, and this has been confirmed by Bedson and Crawford, although it has been denied by others. The serum of highly immunised animals is also capable of conferring passive immunity. Perdrau has found that cerebral immunity to the herpes virus may follow the intradermal inoculation of a neurotropic virus, though if the inoculation is deep (*e.g.* by deep scarification), encephalitis may result. The immunity persists for three months. According to Flexner and Amoss, strains of the virus vary considerably in virulence; those of low virulence only produce encephalitis when inoculated intracerebrally. Further, by inoculating with a mild virus, immunity to the more virulent strains may be produced. There is also evidence that viruses of this type may be present in the saliva of healthy persons (Levaditi, Harvier and Nicolau; Doerr and Schnabel).

A remarkable phenomenon has recently been described by Magrassi and amplified by Doerr and Seidenberg. A rabbit is inoculated intracutaneously on the flank or by scarification of the cornea with a strain of herpes virus which on infection from either of these routes or intracerebrally produces encephalitis. Then after an interval of days, the length of which varies according to the site of inoculation, but which is within the incubation period, it receives an additional inoculation, this time intracerebrally with the same strain of virus. The result is that encephalitis does not develop, the animal remaining in good health and being immune to subsequent reinoculation. If the interval between the inoculations is either too long or too short protection is not obtained and the animal dies of encephalitis. Protection is not obtained if, after corneal inoculation, inactivated formolised virus is used for the intracerebral inoculation. The explanation of this antagonistic effect of the one inoculation on the other is obscure

With regard to the biological nature of the herpes virus little is known. As in epidemic encephalitis, minute granular structures (intracellular and extracellular) have been described in the lesions of the central nervous system, which some observers have regarded as specific and have identified with the virus. These bodies can be demonstrated by Giemsa's stain. The size of the virus bodies is 0·1–0·15 μ. Intranuclear eosinophile inclusions in the epithelial cells of the corneal and skin lesions have been observed by Lipschütz, Goodpasture and Teague, and others. They have also been found in experimental infections in the nerve cells and glial cells of the brain and in the cells of the liver, testis, etc. Similar intranuclear inclusions

are present in the cells of the lesions in herpes zoster. Attempts to cultivate the virus have proved unsuccessful.

Herpes Zoster

The evidence in regard to the infective nature of herpes zoster has been less conclusive than in the case of the other types. Using the vesicle fluid of herpes zoster, Lipschütz succeeded in producing keratitis in a small proportion of the rabbits inoculated, but passage did not succeed. He also described intranuclear inclusion bodies in the lesions, Others have failed entirely to transmit the conditions to animals; this contrasts with the easy transmission of herpes febrilis. In the human subject the production of lesions by inoculation with material from the vesicles in herpes zoster leads to immunity to reinoculation, but does not produce immunity to herpes febrilis. Thus the two viruses are apparently distinct. The virus of herpes zoster and that of varicella have been held to be closely related if not identical; the evidence for this is discussed under Varicella (*vide infra*). Elementary bodies have been demonstrated in the vesicle contents (Paschen, Taniguchi and others).

Varicella (Chicken-pox)

The inoculation of animals with material from cases of varicella has, as a rule, yielded negative results; but Rivers has recorded in African vervet monkeys after intra-testicular injection the local occurrence of nuclear inclusions in endothelial leucocytes and less commonly in the tubule cells of the injected testis, which are similar to those met with in the lesions of the human disease. Elementary bodies have been demonstrated in the vesicle contents (Aragão, Paschen, Amies, etc). Confirmation of the above work has come from Taniguchi and others, who have obtained growths of elementary bodies in tissue cultures and on the chorio-allantoic membrane of the chick. Amies has found that from five days after the onset of the disease antibodies appear in the serum which agglutinate the elementary bodies, which he got by centrifuging fluid from the vesicles.

The occurrence of cases of varicella along with cases of herpes zoster has been observed, and the relation has been supported by the experiments of Kundratiz. He found that the inoculation of the skin of children with fluid from the vesicles of herpes zoster led in about two-thirds of the cases to the pro-

duction of local vesicles ; this was followed in some by a general eruption indistinguishable from that of chicken-pox, and the development of a general reaction without a local one was also observed. Further, children who had previously had chicken-pox were insusceptible to inoculation with herpes zoster material. Also those who had reacted to the herpes zoster virus were found later to be insusceptible to inoculation with varicella material. Cross agglutination and complement-fixation reactions have yielded results confirmatory of the above findings as to the close relation of the viruses of varicella and herpes zoster (Brain, Amies). There appears not to be a complete antigenic identity, however.

Morbilli (Measles)

The earliest reliable experimental observations on the etiology of this disease were made by Hektoen in 1905, who reproduced measles in volunteers by the subcutaneous injection of blood from cases at an early stage of the illness. No bacteria could be demonstrated in the blood by culture. Anderson and Goldberger, a few years later, were apparently successful in transmitting the disease to monkeys by inoculation with blood and with nasal and pharyngeal secretions. They found that the causal agent was filterable through Berkefeld filters, and they demonstrated its presence in the blood and throat secretions of inoculated animals. Though some workers have been unable to confirm these results, it seems now well established that a virus is present in the blood at an early stage, which is experimentally transmissible to monkeys, reproducing a condition similar to human measles. Blake and Trask inoculated monkeys (*Macacus rhesus*) intratracheally with filtered naso-pharyngeal washings from cases at the commencement of the eruptive stage, and produced a condition presenting many similarities to human measles, with lesions of the skin and the mucous membrane of the mouth corresponding histologically to the measles eruption. They were able to pass the infection through a series of animals, and they demonstrated a specific immunity following an attack. Taniguchi and his co-workers have succeeded in establishing in rabbits several strains of virus from the blood and filtered naso-pharyngeal washings of measles patients. When the virus has been adapted to rabbits after repeated intra-testicular passages orchitis develops. On transfer of the virus from rabbits to susceptible children, typical attacks of measles have been produced. Also, the serum of convalescent patients has a

neutralising action on the virus. Elementary bodies have been demonstrated in smears from the cornea of rabbits after inoculation into the anterior chamber of the eye.

Tunnicliff and others have described Gram-positive diplococci of the "viridans" group which have been cultivated from the blood and the nasal, pharyngeal, and conjunctival secretions in the early stage of measles. According to Park, Williams and Wilson, however, such cocci recovered from cases of measles are of various types, and they could not, by means of their toxins, distinguish between persons susceptible to measles and those immune. The causal relationship of the anaerobic Gram-negative diplococcus isolated by Caronia has not been confirmed.

The immunity following an attack of measles has long been recognised, and experimental work has shown that it depends mainly on specific properties resident in the blood serum (Nicolle and Conseil). The serum of convalescents, taken one week after defervescence has been used both in the prophylaxis and treatment of measles by Degkwitz and others, with promising results. It has been found that if the immune serum is injected during the first five days after infection, an effective passive immunity is produced which may persist for a month. According to Brincker the dose for a child is double the number of c.c. corresponding to its age in years, injected intramuscularly. Further, an injection even after the fifth day, *i.e.* on the seventh to tenth day, tends to modify the subsequent attack; and this procedure may be adopted with advantage, as suggested by Debré and Ravina. The resulting mild attack of the disease confers a lasting active immunity. With a view to providing facilities for the supply of convalescents' serum, centres have been established in various countries for collecting and distributing the serum, which is obtained from convalescent donors over ten years of age (it is necessary, of course, to ensure that they are free from any other systemic infection, *e.g.*, syphilis, tuberculosis). The pooled serum of usually three persons is used, but at present there is no means of standarising the serum. The use of normal adults' serum in a dose double that of convalescent serum has also been recommended. Further, preparations of globulins extracted from human placentas have been stated to contain the antibody and to be an effective prophylactic agent.

Infective Parotitis (Mumps)

It has been established by the work of Granata and others that rabbits, monkeys, and cats develop lesions when inoculated

with sterile filtrates of the saliva from cases of mumps or of fluid obtained by puncturing the parotid gland. The disease can be transferred in series through cats by means of inoculation into the parotid gland or testis (Wollstein). Swelling and tenderness of the inoculated organs develop after five to nine days, and the microscopic changes are similar to those in the human disease. The virus disappears from the saliva between the sixth and ninth day of the disease; it is present in the blood in severe cases. Blood serum from a recovered animal when mixed with tissue containing the virus neutralises the action of the latter. After intracerebral inoculation of filtered mouth washings from acute cases of mumps into monkeys, meningitis and degenerative changes in the nerve cells of the brain and cord occur, and there are changes in the parotid when survival is sufficiently prolonged (Gordon). The disease can be produced in monkeys by introducing infective filtrates into Stenson's duct and a series of passages have been effected in this way. The virus survives in 50 per cent. glycerol for at least five weeks.

According to Kermorgant, the causal organism is a spirochæte, which he grew anaerobically in mixed cultures with mouth bacteria; and he claims to have reproduced the disease with the organisms even when they had been subcultured over a period of ten months. Infective filtrates when inoculated with the bacteria yielded mixed growths containing the spirochæte Also it is stated that the serum in convalescence contains agglutinins and lysins for the spirochæte.

Epidemic Influenza

The inconstancy of Pfeiffer's bacillus in epidemic influenza, the fact that indistinguishable organisms may occur in various inflammatory conditions of the respiratory system, and the absence of experimental proof of its etiological relationship to the disease, led to further inquiry being made into the etiology of influenza during the pandemic of 1918 and the following years. The knowledge that had been obtained regarding the causation of certain specific infectious diseases by filter-passing viruses directed attention particularly to the question of whether this epidemic disease was the result of infection by such a virus. Further, the extreme infectiousness of the condition, by analogy with other conditions known to be due to filterable viruses, seemed to favour this view of the etiology of the disease. In the early stages of the pandemic, various workers claimed to have established the existence of a filter-passer in the disease (Gibson, Bowman and Connor; Bradford, Bashford and

Wilson; Nicolle and Lebailly; Yamanouchi, Sakami, and Iwashima; and others). Certain of these claims, however, were subject to serious criticism in regard to the technique of the experimental work on which they were based, though the Japanese workers stated that they produced an influenza-like disease in susceptible persons by the injection into the nose and throat of filtrates of sputum and blood from influenza cases.

In recent years further light has been thrown on the etiology of influenza by work at the National Institute for Medical Research, London (Laidlaw and his co-workers). During an epidemic in 1933 filtered throat washings obtained from patients at an early stage of the illness were found to be infective to ferrets by intranasal instillation. No other laboratory animals could be similarly infected. Illness ensued in the ferret after an incubation period of two days and showed characteristic features, namely, a diphasic temperature response, nasal catarrh and systemic disturbances. The temperature at the onset rose abruptly, subsided on the third or fourth day and rose again on the fourth or fifth day, later gradually returning to normal. With the rise of temperature the animals became lethargic and showed muscular weakness. The catarrh usually began on the third day with a watery discharge from the eyes and nose. The nasal discharge tended to become muco-purulent. Frequent sneezing was a prominent symptom. Signs of illness lasted as a rule for only a few days and complete recovery then ensued. Very occasionally a relapse was observed. No fatalities resulted. In animals killed during the first and second febrile periods the mucosa of the nasal passages showed acute inflammation with complete disappearance of ciliated cells and occasionally patchy necrosis of the whole thickness of epithelium (Smith, Andrewes and Laidlaw).

It was found that the disease could be transmitted by contact or by transference of nasal washings from infected to normal ferrets. The method adopted for transmission experiments was to scrape out the turbinates and grind up the tissue with sand and then emulsify in a mixture of broth and saline. The emulsion was centrifuged and 1 c.c. of the fluid was instilled into the nose. In this way it was possible to make serial passages. No other method of inoculation proved successful and no other tissue, with the exception of the lungs, was found to be infective. Filtrates of these emulsions obtained with gradocol membranes having an average pore-size of 0·6 μ invariably produced the typical disease. No bacteria

were cultivated from the filtrates. Ferrets which had recovered from the illness were in all cases immune to subsequent inoculation. The serum of recovered animals also neutralised the virus when mixed with it and the mixture inoculated into the nose. Normal ferret serum had no such action. Human sera, particularly those from influenza convalescents, were also found to contain neutralising antibodies for the virus. It may be noted that throat washings from healthy persons and influenza convalescents caused no illness in ferrets.

Previous to these observations Shope in the United States had described a filterable virus in an influenzal disease of pigs, but found that it was associated with a hæmophilic organism (*B. influenzæ suis*, p. 585). By experiment he showed that the virus *per se* produced in pigs only a mild illness, while the bacillus was inactive. On the other hand the two agents together caused the typical disease. The workers at the National Institute found that the swine influenza virus of Shope caused an illness in ferrets similar to that due to the human influenza virus. Shope found later that the swine influenza virus when introduced intranasally in ferrets under ether anæsthesia produced often extensive atelectasis and consolidation of the lungs.

Attempts were made by the National Institute workers to adapt the virus from ferrets to mice and this was found possible, the virus material being instilled into the nose under ether anæsthesia. After several passages in mice, the virus infected nearly all mice so inoculated and frequently led to a fatal illness characterised by pneumonia. After passage through mice the virus was still virulent to the ferret. The *upper* respiratory passages in the mouse did not appear to be susceptible as in the ferret. The same results were obtained with Shope's virus. It has not been possible, however, to infect mice directly from the human subject. It was estimated by filtration through Elford's gradocol membranes that the diameter of the virus bodies was from 0·08 to 0·12 μ.

Virus has now been isolated by these methods on several occasions from epidemic influenza in Great Britain and America, and different strains have proved to be neutralisable by one specific antiserum, although minor differences are detectable.

The condition of swine influenza is of special interest in connection with the etiological problem of the human disease. This infection was apparently unrecognised before 1918 when it was first noticed—in the United States—simultaneously with

the occurrence of the human pandemic of influenza at that time; since then it has persisted. The symptoms and signs of the disease distinctly resemble human influenza. Reference has already been made to the virus of this disease and its pathogenicity in association with an influenza bacillus. The question therefore arose whether this virus is identical with strains of human origin. The National Institute workers have shown by neutralisation tests with antisera that while the antigens of the two viruses have common constituents, they are not completely similar. Laidlaw has suggested that the virus of swine influenza is in reality the virus of the pandemic disease of 1918, adapted at that time to the pig and maintained since then in this animal. The further question arises whether the virus of the 1918 pandemic was antigenically different from that now present in influenza, and whether influenza viruses may vary in antigenic characters under different conditions and at different times. Only continued investigation in the course of time will settle this question.

An anti-influenzal serum has now been obtained by Laidlaw and his co-workers by hyper-immunisation of horses with virus emulsions from infected ferrets. Such serum, even in high dilution, inactivates the virus and can confer some degree of passive immunity in mice even after they have already been infected. Immunity to the disease in ferrets has also been studied further: after recovery ferrets remain resistant for at least three months and thereafter the immunity slowly declines. At this stage, although antibodies are still present in definite amount in the serum, the nasal mucosa is susceptible to infection. Formolised virus is capable of inciting active immunity.

Disease has been produced in young pigs by intranasal inoculation with the human virus (Elkeles, Shope and Francis). The illness is of a mild type, but there may be broncho-pneumonic areas in the lungs or lobular atelectasis. According to Shope and Francis, inoculation of pigs with a mixture of this virus and *B. influenzæ suis* brings about a more severe illness attended with fever and pneumonia.

Such observations have now defined the virus of epidemic influenza and have opened the way to possible advances in the diagnosis and control of this infection. Further light has also been thrown on the relationship of *B. influenzæ* to the disease and the part it may play in intensifying the lesions in the respiratory system.

The virus has been cultivated *in vitro* in chick-embryo tissue

cultures, and it has been found that the cultivated virus when inoculated subcutaneously or intradermally in the human subject is non-infective, but stimulates the production of specific antibodies. Thus the serum of inoculated persons is capable of protecting mice against the virus (Francis and Magill). A formolised vaccine has also been prepared by the National Institute workers from infected mouse lungs. So far it has not been possible to ascertain whether such preparations have a prophylactic action against the epidemic disease.

Infectious Coryza (the "Common Cold")

The infectious nature of this prevalent condition is now well recognised, and the infection can frequently be traced to contact with other cases of the same condition, the incubation period being within three to four days. The condition is characterised by a preliminary dryness of the nasal mucosa, often with sneezing, followed by an abundant watery discharge from the nostrils; there may be some degree of malaise, headache, and, in some cases, slight fever. After two or three days the discharge becomes less, and tends to assume a purulent character; restoration to normal then occurs after a varying number of days. It is to be noted that in the purulent stage, secondary spread may occur, *e.g.* to the nasal sinuses, middle ear, and at this stage various pyogenic organisms commonly found in inflammatory conditions of the upper respiratory passages may be present, *e.g.* staphylococci, streptococci, pneumococcus, *B. influenzæ*, *M. catarrhalis*, etc.

In 1914, Kruse claimed that the condition was due primarily to a filterable virus, and he was able to reproduce the condition in volunteers after an incubation period of one to four days, by introducing into the nose the filtered nasal secretion from a case. The filtrate, obtained by means of a Berkefeld filter, was apparently free from any recognisable organism. Foster claimed to have confirmed these results. Olitsky and McCartney, using as the inoculum naso-pharyngeal secretions from early cases in the first three to eighteen hours of the disease, the material being filtered through Berkefeld V and N filters, succeeded in transmitting the condition to volunteers, and further, were able to pass it from an experimentally infected case. Intratracheal injection in rabbits both with filtered and unfiltered naso-pharyngeal washings was without effect. These observers were unable to isolate by culture in the

Smith-Noguchi medium, and on anaerobic blood-agar plates, any specific cultivable organism.

Shibley, Mills and Dochez, having observed that young chimpanzees readily develop nasal catarrh on contact with affected persons, could reproduce the condition in a large proportion of these animals inoculated by instilling into each nostril fresh, filtered nasal washings from human cases at the acute stage. The incubation period was thirty-six to forty-eight hours. The filtrates after heating were ineffective, as were also unheated filtrates from nasal washings of normal persons obtained at a time when coryza was not prevalent. On several occasions the condition was transmitted in series to further animals ; others also contracted catarrh by contact with those affected. An important observation was that coincidently with the appearance of symptoms, pathogenic types of organisms such as the pneumococcus, *Streptococcus hæmolyticus,* and Pfeiffer's bacillus increased greatly in the naso-pharynx, and they appeared in the nose, where they seldom occur normally in apes. Immunity was found to last three to four months. During an influenza epidemic recovered animals, which were in the refractory period, contracted a more severe condition, as regards constitutional and respiratory symptoms, from contact with a keeper. This suggested that influenzal infection is distinct from that of the common cold. Dochez, Mills and Kneeland succeeded in cultivating *in vitro* the virus of the common cold, by a method previously applied in the cultivation of certain other filterable viruses : they used a medium consisting of a buffered broth containing an emulsion of chick-embryo tissue and with 1 : 2000 cysteine added to yield a suitable reduction potential. They were able to reproduce colds in volunteers by inoculation with the cultivated virus even after fifteen subcultures. It has been possible to cultivate the virus artificially for long periods, up to one year.

Mixed vaccines prepared from the common organisms found in coryza (*vide supra*) have been extensively used with a view to prophylaxis. Carefully controlled tests of the efficacy of these vaccines have been made on a statistical basis (Jordan and Sharp ; Ferguson, Davey and Topley), but the results have not established any definite prophylactic properties.

From these observations it would appear that the common infectious " cold " is primarily due to a transmissible filterable virus, which may predispose to secondary infection with pyogenic bacteria.

Acute Rheumatism

It was pointed out by Schlesinger, Signy and Amies that the deposit obtained by high-speed centrifuging of pericardial fluid from cases of acute rheumatic pericarditis contained particles morphologically resembling virus elementary bodies (they were also got in the pleural exudate in one case). Barnard has estimated their size to be between 80 and 100 mμ. Suspensions of these bodies prepared in formol-saline were specifically agglutinated by the sera of patients who were suffering from, and successfully resisting, acute rheumatic infection. The sera of patients in the quiescent stage of the disease as a rule failed to agglutinate the bodies. Also, serum of normal persons and of patients with various non-rheumatic infections failed to agglutinate them. It was suggested that these bodies may represent the actual infective agent of acute rheumatism, and that streptococcal infection (p. 307) plays a part either by lowering resistance and enabling the virus to enter the tissues or, if it is already present in a latent state, in allowing it to become active. Cross agglutination points to rheumatic fever, chorea and rheumatoid arthritis being etiologically related (Eagles and others).

Psittacosis

The occurrence of cases of serious human illness with the features of pneumonia and typhoid, in association with gastro-intestinal disease in parrots has long been known. A bacillus isolated from the bone marrow of dead parrots in Paris by Nocard (1892) was held to be the causal agent, but has since been identified as *B. aertrycke*, and has been proved to have no connection with the disease. In 1929 numerous cases of psittacosis appeared in the Argentine, the United States, Great Britain, and various parts of the Continent. The great majority of these were obviously related to diseased parrots recently imported, and when importation was prohibited the epidemic soon ceased. The characteristic lesion in human psittacosis is a widespread pneumonic consolidation, which to the naked eye may resemble that found in influenzal pneumonia, but the microscopic appearances are different. In psittacosis pneumonia there are found in the alveoli marked fibrinous exudate and some hæmorrhage, with mononuclear cells, but relatively few polymorphs ; in the most advanced parts of the lesion there is also extensive capillary thrombosis. Influenza bacilli and the other organisms commonly found in pneumonia are not present.

Bedson, Western and Levy-Simpson demonstrated that the disease could be transmitted to budgerigars (*Melopsittacus undulatus*) by intraperitoneal injections of sterile filtrates of the tissues of infected animals. Repeated passage has been effected,

but the virulence of the infection tends soon to become extinguished in these birds. Fowls are susceptible, but passage cannot be effected. Parrots can be infected by the mouth or nose as well as parenterally. Mice are also susceptible to inoculation with infective material from birds, but apparently not to such material when directly derived from man. Passage of the infection succeeds in mice also, and finally a constantly fatal result follows intraperitoneal inoculation with liver and spleen emulsion. Levinthal and others have demonstrated the presence in the disease of minute cocci or cocco-bacillary bodies about 0·2 μ in size, stained purple to blue by prolonged treatment with Giemsa's stain or blue by the use of Castaneda's stain. These bodies, which are Gram-negative, occur especially in the spleen and liver in affected birds, but are scanty in the blood. The bodies are situated within endothelial cells, but occur also in clumps lying free; they have been seen in similar cells from the pneumonic areas in man. The bodies show great pleomorphism, the size varying from 1 μ down to minute particles, and a sequence of changes may be made out. Bedson and Bland have interpreted the appearances as indicating a developmental cycle. They have not been grown on ordinary media. Their number in a tissue appears to correspond with its virulence for susceptible animals. The virus is present in the nasal secretions and fæces of infected parrots, as well as in the blood and internal organs. Its infectivity for man is so high that the disease may be contracted without close contact with affected birds being traceable. The occurrence of pneumonia as the chief lesion in man seems to point to the disease being acquired by inhalation; this is supported by the production of similar lesions in the lungs of monkeys following intranasal or intratracheal inoculation (Rivers and Berry), whereas intracerebral inoculation in these animals leads to a meningo-encephalitis, but pneumonia does not develop. Parrots, however, by whatever route they are infected, do not show lesions of the lungs, the duodenum and the liver being chiefly affected. The virus has been found in the sputum of a human case, which suggests that the disease may be directly contagious; clinical observations are in agreement with this, but such spread appears to occur only rarely. Both in birds and in man the severity of the disease may vary greatly, and the evidence points to the existence among parrots of apparently healthy carriers of the virus. In rabbits intracutaneous inoculation leads to a local papular eruption resembling that which may be produced by the virus of herpes, while intracerebral

inoculation causes meningo-encephalitis (Gordon). Recovery is accompanied by active immunity. The psittacosis virus is not influenced by an antiserum to fowl-plague virus; this observation, as well as the fact that the former is infective for mice but not the latter, indicates the separate identity of the two viruses.

Diagnosis.—In *birds* suggestive lesions are pericardial effusion, greatly enlarged spleen, pale liver with necrotic areas sometimes with hæmorrhagic periphery. Smears from the pericardial and peritoneal exudates, blood, lungs, and serous and cut surfaces of liver and spleen (particularly from necrotic areas) are stained for virus bodies. Several mice are inoculated intraperitoneally with 0·5–1·0 c.c. of a mixed emulsion of the various tissues and also with the same material after clarification and filtration through a coarse type of bacterial filter, *e.g.* Berkefeld V—the latter procedure being necessary in case there is a mixed infection with pathogenic bacteria. In the *human* subject the virus may be found by inoculating mice with defibrinated blood (0·5–1·0 c.c.) in the first four days of the illness, but is more likely with pleural effusion or sputum. The latter is emulsified in 10 volumes of broth by shaking with glass beads, and is inoculated unfiltered and also after clarification and filtering. *Post mortem*, material obtained at a late stage of the illness is usually negative, but scanty virus may be detected by giving a mouse three injections on successive days.

Infected mice die usually in five to thirty days. If death has occurred in the first half of this period virus particles are readily found in the endothelial cells of the peritoneal and pericardial effusions; later they are scanty. Passages through further mice raise the virulence till death occurs in two or three days, virus bodies being abundant in the spleen phagocytes. If all the mice originally inoculated survive for ten days, some should be killed at this time and their spleens used to inoculate further animals. If the rest of the original mice survive for a month they should be treated similarly.

Great care should be taken with all animals and materials to avoid dissemination of infective dust. Carcases before examination should be soaked in a solution of liq. cresol. sap so as to thoroughly wet hair or feathers.

Yellow Fever

Yellow fever is an infective disease which is endemic in the West Indies, in Brazil, in Sierra Leone and the adjacent parts of West Africa. From time to time serious outbreaks take place, during which neighbouring countries also suffer, and the disease may spread to other parts of the world. In this way epidemics have arisen in the United States and elsewhere. In the parts where it is endemic, though usually a few cases may occur from time to time, there is some evidence that occasionally the disease may remain in abeyance for many years and then originate

apparently *de novo*. It is possible that continuity may be maintained by the persistence of a mild type of the disease, which may be grouped with the "bilious fevers" prevalent in yellow fever regions. This would explain the degree of immunity which is shown during a serious epidemic by the older inhabitants. Till recently yellow fever was regarded as being pre-eminently a disease of urban populations, but it has recently been discovered to exist in country districts in Brazil.

Great variations are observed in the clinical types under which the disease presents itself. Usually after from two to six days' incubation a sudden onset in the form of a rigor occurs. The temperature rises to 104°–105° F. The person is livid, with outstanding bloodshot eyes. There are present great prostration, pain in the back, and vomiting, at first of mucus, later of bile. The urine is diminished and contains albumin. About the fifth day an apparent improvement takes place, and this may lead on to recovery. Frequently, however, the remission, which may last from a few hours to two days, is followed by an aggravation of all the symptoms. The temperature rises, jaundice is observed, hæmorrhages occur from all the mucous surfaces, causing, in the case of the stomach, the "black vomit"—one of the clinical signs of the disease in its worst form. Anuria, coma, and cardiac collapse usher in a fatal issue. The mortality varies in different epidemics from about 35 to 99 per cent. of those attacked. Both white and black races are susceptible, but those who have resided long in a country are less susceptible than new immigrants. An attack of the disease usually confers complete immunity against subsequent infection.

Post mortem the stomach is found in a state of acute gastritis, and contains much altered blood derived from hæmorrhages which have occurred in the mucous and submucous coats. The intestine may be normal, but is often congested and may be ulcerated; the mesenteric glands are enlarged. The liver is in a state of fatty degeneration of greater or less degree, but often resembling the condition found in phosphorus poisoning. The kidneys are in a state of intense glomerulo-nephritis, with fatty degeneration of the epithelium. There is congestion of the meninges, especially in the lumbar region, and hæmorrhages may occur. The other organs do not show much change, though small hæmorrhages under the skin and into all the tissues of the body are not infrequent. In the blood a feature is the excess of urea present, amounting, it may be, to nearly 4 per cent.

Histological changes in the Liver.—The changes are highly

characteristic. There is a hyaline necrosis of the liver cells throughout the lobules, but most pronounced in the middle zone; along with this there is marked fatty change of the surviving parenchyma in the central and peripheral zones and in the non-necrotic cells situated among the necrotic ones. The absence of hæmorrhage, inflammatory changes or collapse of the lobular structure is also a marked feature. An instrument of the nature of a large trocar, the "viscerotome," has been devised by means of which specimens of liver tissue can be readily obtained for histological examination in order to confirm or exclude yellow fever in cases which have died from a suspicious illness, without the necessity for an autopsy.

Etiology and Transmission.—The earliest bacteriological work on the etiology of yellow fever is now only of historical interest. Long before the actual virus of the infection came to be investigated, it was suspected that a mosquito acted as the intermediary host, and in 1881 Finlay claimed that *Stegomyia fasciata* (now designated *Aëdes ægypti* or *calopus*) was the vector of the infection. In 1900 a United States Army Commission tested this hypothesis. Selecting mosquitoes which they had reared from eggs, they allowed them to bite yellow fever patients, and then to bite healthy men. Of several experiments of this nature two were successful in the first instance, the first individual to be infected in this way being Dr. James Carroll, a member of the Commission, who passed through a severe attack of typical yellow fever. Experiments were then performed on a larger scale, with completely confirmatory results as to the conveyance of the disease by mosquitoes. Of twelve non-immunes living under circumstances which excluded natural means of infection, ten contracted yellow fever after having been bitten by mosquitoes which had previously bitten yellow fever patients; happily all of these recovered. Two of the men who were thus infected had been previously exposed to contact with fomites from yellow fever patients without results. These results were confirmed by Guitéras. whose investigations were carried out along similar lines; of seventeen individuals bitten by infected mosquitoes, eight took yellow fever, and three of these died.

The species of mosquito used by the American Commission in Cuba was the *Aëdes ægypti*. Recently in West Africa it has been shown also that several other species of *Aëdes* can transmit the infection. It has been determined that a certain period must elapse after the insect has bitten a yellow fever patient before its saliva becomes infective to another subject. The

length of this period depends on the temperature. It may be as short as nine days ; at 22° C. it is at least three to four weeks ; and at 20° C. the virus does not develop. The virus, however, can be shown to be continuously present in the body of the mosquito from the time it has ingested the infective blood. Infected mosquitoes retain the power of infection for a considerable time afterwards—ninety days or longer. It has also been shown that mosquitoes may become infective after biting a patient on the first, second, or third day of the disease, but at a later period the results are usually negative, apparently because the virus is no longer present in the blood. Hereditary transmission of the virus through the mosquito's eggs has not been determined.

Interesting results were also obtained with regard to the communication of the disease directly from patient to patient, the conclusion arrived at, after careful experiments, being that the disease cannot be transferred in this way, even when the contact is of a close character. In a specially constructed house seven men were exposed to the most intimate contact with the fomites of yellow fever patients for a period of twenty days each, the soiled garments worn by the patients being in some cases actually slept in by these men ; the result was that not one of those thus exposed contracted the disease. The conclusions on this point have been subsequently confirmed by other workers.

The American Commission also found it possible to transmit yellow fever to a healthy man by injecting small quantities of blood or of serum taken from a yellow fever patient at any period up till the third day of the disease. The period of incubation in this case is somewhat shorter than when the disease is conveyed by the bite of mosquitoes, the average duration in the former case being about three days, and in the latter about four days, though these times may be considerably exceeded. The blood or serum used in these experimental infections was found to be free from bacteria. That the blood is highly infective in the early stage is shown by the fact that the disease has been contracted in the course of the manipulations incidental to making a count of a patient's blood (Low and Fairley).

Experiments with regard to the nature of the yellow fever virus were carried out by Reed and Carroll ; they found that it was very easily killed by heat, as blood from a yellow fever patient lost its infective power on being heated to 55° C. for ten minutes (on the other hand, it is highly resistant to freezing). Blood or serum was found to be still infective after having been

passed through a Berkefeld filter. This was confirmed by the French Commission, with the additional result that the virus passed through a Chamberland filter (F grade), but not through one of a finer grade (B). These facts led to the classification of the parasite among the so-called ultramicroscopic or filterable viruses. By filtration the size of the virus has been estimated at 17–28 mμ (Findlay and Broom). Attempts to demonstrate the organism by microscopic methods have failed.

Noguchi in 1918 in Guayaquil isolated a leptospira, which has now been shown to be indistinguishable from that of Weil's disease (p. 700). This organism has not been found in the cases investigated in West Africa, and, as will be seen, the evidence points to yellow fever being of the same nature in America and Africa.

The discovery by Stokes, Bauer and Hudson, working in West Africa as members of a Commission of the Rockefeller Foundation, that certain Asiatic monkeys, especially *Macacus rhesus*, are highly susceptible to the disease has led to further important advances in knowledge regarding the behaviour of the virus and immunity phenomena. Susceptible monkeys may be readily infected by subcutaneous or intraperitoneal injection of material containing the virus, *e.g.* the blood of a patient in the first three days of the disease, or even by smearing infected blood on the intact skin; they may be infected also by the bites of infected mosquitoes. After an incubation period, which varies somewhat according to the method of inoculation but is usually three to four days after the bite of infected mosquitoes, marked pyrexia develops, and then albuminuria with casts and bile in the urine, and jaundice. Bleeding from the gums and black vomit occasionally occur. The infection can be maintained by passage from animal to animal by inoculation of blood or an emulsion of liver. The virulence of the virus appears to vary, but in the case of *M. rhesus* monkeys with a recently recovered virus the result was almost invariably fatal, death occurring usually six to ten days after inoculation. The pathological changes closely resemble those in the human disease. Acidophile intranuclear inclusions have been described in the liver cells in monkeys infected with yellow fever by Torres and others (Fig. 173), although they are rare in man. The infective agent is abundant in the blood or serum of affected monkeys, since 0·0001 c.c. injected subcutaneously into a susceptible animal will usually transmit the disease. It has been proved by inoculation of monkeys that cases of mild febrile illness which could not be diagnosed clinically as yellow fever, contained the virus in their blood. In fact, it was from

mild cases that the disease appeared to be most readily conveyed to monkeys. As a rule the virus has not been found in the organs *post mortem* in human cases. African monkeys, guinea-pigs, and other animals appear to be insusceptible, but the hedgehog is highly susceptible (Findlay and Clarke). Theiler has shown that the virus can be passed in series through mice by intracerebral inoculation. As a result of such repeated brain passages a "neurotropic" strain is produced which on inoculation into monkeys subcutaneously is found to have lost the original "viscerotropic" characters. This neurotropic virus causes encephalitis when injected intracerebrally into

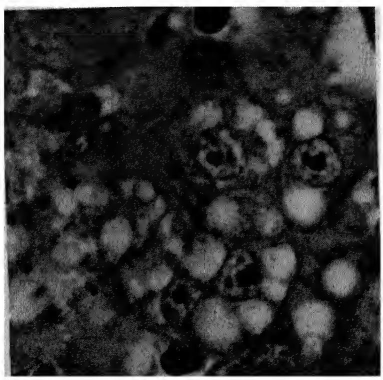


FIG. 173.[1]—Section of liver from monkey experimentally infected with yellow fever. Inclusions (Torres bodies) are seen within the nuclei of the liver cells: there is also extensive fatty degeneration with necrosis. Hæmatoxylin and eosin. ×950.

mice, monkeys (including species insusceptible to viscerotropic virus), and certain other animals. Injection of the neurotropic virus directly into the liver of monkeys rapidly restores its original viscerotropic property (Findlay and Clarke). Multiplication of the virus has been obtained in a medium resembling that used for vaccinia, but containing mouse embryo instead of chick embryo.

Jungle Yellow Fever.—Soper and others have discovered that in Brazil, yellow fever exists in country districts and in the absence of *Aëdes ægypti*. Mosquitoes of the genus *Hæmagogus*

[1] We are indebted to Dr. G. M. Findlay for the preparation from which Fig. 173 was taken.

have been shown to be the vectors. The existence of the disease in sparsely populated districts suggested an animal reservoir. The facts that the infection can be transmitted to monkeys by the bite of mosquitoes, and that monkeys caught in South America have antibodies to the virus in their serum point to these animals as the natural source of infection.

Immunity.—Recovery from yellow fever is followed in monkeys as in man by immunity. The serum of such immune individuals, even in small amounts, confers passive immunity against the virus. On the other hand, the serum of the naturally insusceptible African monkeys and of individuals who have not been exposed to infection does not possess this property; hence the presence of protective powers in the serum may be taken as evidence that the person has had an attack of yellow fever. By cross immunity tests in monkeys it has been shown also that the yellow fever of South America and West Africa are identical, since recovery from the one infection is followed by resistance toward infection with the other. Similarly the serum of recovered individuals confers passive immunity toward either virus. The serum of insusceptible animals, *e.g.* horses, which have been injected with the virus acquires anti-infective properties, as is shown by mixing it with a fatal dose of the virus and inoculating a susceptible animal. Such antiserum, as well as that of recovered cases, has been used for curative purposes both in the natural and the experimental disease, but so far only slight results have been obtained.

The fact that inoculation with virus modified in virulence, gives rise to antiviral properties in the serum has led to several methods of prophylactic inoculation in the human subject. The virus used has been either the neurotropic strain or a pantropic virus attenuated by prolonged cultivation *in vitro*, and the innocuousness of such vaccines has been stated to have been proved. But in order to avoid risks in susceptible subjects, either convalescent human serum or an immune serum from horses has been injected along with the living virus. The efficacy of this method has been proved by the immunity to yellow fever shown by laboratory workers thus protected, whereas the disease had previously been frequently contracted by those engaged in investigating it.

The nature of the immunity following recovery has not been fully explained, since liver or spleen of an actively immunised animal when perfused till free from blood is capable nevertheless of producing immunity when injected into a susceptible monkey (Hindle). Immunisation with the virus appears under certain conditions to produce supersensitiveness to the disease; thus a

further injection of a large amount of virus administered several months after a previous dose may lead to death of monkeys within thirty-six to forty-eight hours with symptoms resembling those of yellow fever. *Post mortem* the organs are free from virus, so that the effect appears to be of toxic nature.

Sawyer and Lloyd have devised a method of testing serum for virus-neutralising properties which depends on the use of mice The serum is added to a suspension of the brains of mice infected with the neurotropic virus and the mixture is injected intraperitoneally into a mouse; at the same time a small amount of boiled starch solution is injected intracerebrally, so as to localise any unaffected virus. If the serum lacks neutralising power, death occurs from encephalitis. Frobisher has obtained a complement-fixation reaction with the serum of convalescents from yellow fever, using as antigen an extract of monkey's organs containing the virus. Accordingly, the method may prove of use in the diagnosis of recovered cases which have not been clinically characteristic.

Dengue

Dengue is a condition presenting a fairly definite clinical picture and has been long known to have an extensive geographical distribution. The fever lasts about six days, but the period varies; there is a terminal skin eruption, which is characteristic and which does not occur in phlebotomus fever or the other febrile illnesses met with in tropical and subtropical countries. The virus disappears from the blood after the first few days. Ashburn and Craig, working in the Philippines, found the blood in this disease to be infective after filtration through a Berkefeld filter, and this has been confirmed.

No animal has been found susceptible, but Blanc, Caminopetros, and Manoussakis showed that the blood or serum of guinea-pigs five days after an injection of infective blood may still harbour active virus as tested by inoculation of the human subject. The virus survived similarly in various other animals. Infectivity is retained for some days when blood containing the virus is kept at room temperature, but is destroyed in half an hour at 50° C. Epidemiological evidence points to the infection being transmitted naturally by the bites of *Aëdes ægypti*, the mosquito which also conveys yellow fever; and this has been verified experimentally. As in the case of the latter disease, the infectivity of the female insect develops after an incubation period which is over a week, provided that the temperature is sufficiently high (20° C.); and then it persists throughout life. Apparently, however, the insect may be infective at an earlier date, and this has raised the question as to whether there may not be several modes of transmission. The

virus is not hereditarily transmitted in the infected mosquito's eggs. Attempts to convey the infection by *Culex fatigans* have, as a rule, failed. Recovery leads to immunity which may, however, be of short duration, as a second attack has developed less than two months after the first. Attempts to demonstrate anti-infective properties in the serum of convalescents have failed; also an antiserum which neutralises the virus of yellow fever is without action on that of dengue. The causal agent has not been identified. Couvy reported the isolation of a leptospira from cases in Beirut; but Kligler and Ashner in Jerusalem failed to find spirochætes either in material examined directly after prolonged centrifuging or by cultivation. Also no characteristic organisms have been found in infective mosquitoes. It would appear that, as in the case of yellow fever, there is liability to confusion clinically between certain types of Weil's disease and dengue and that the same applies to phlebotomus fever.

Rift Valley Fever

This disease was described in 1931 by Daubney, Hudson and Garnham in Kenya. It affects sheep chiefly, causing a high mortality among lambs, but cattle and goats are also affected. It has been transmitted to the human subject by inoculation with a filtrate of infected tissue; a number of laboratory workers also have contracted the disease from contact with infected animal material. In man the attack resembles that of dengue. The virus is present in the blood for nine days after the onset. In sheep the characteristic lesion found *post mortem* is focal necrosis of the liver, and intranuclear acidophile inclusions have been found in the liver cells. The virus is widely distributed in the tissues; it survives *in vitro* for many months at 4° C. in a mixture of oxalated blood and glycerol. Proliferation occurs in a medium of chick embryo and Tyrode solution and also on the chorio-allantoic membrane of hens' eggs. Filtration experiments indicate that the virus particles have a size of 23–35 mμ. Monkeys and various rodents, including rats and mice are susceptible. In mice death occurs in two to four days after inoculation by various routes. The virus can pass through the placenta to the embryo. Recovery is followed by the appearance of protective properties in the serum, and cross immunity tests have shown that the virus is distinct from that of yellow fever and dengue (Findlay). The natural vectors are probably mosquitoes.

Phlebotomus Fever

In the Adriatic littoral there occurs a disease known as "pappataci," characterised by fever and pains in the muscles lasting for about three days, followed by somewhat prolonged prostration, but very rarely having a fatal issue. A similar disease is found widely throughout tropical and subtropical countries. Doerr, after failing to demonstrate any organism in the blood, found that the subcutaneous injection of from 0·5 to 1 c.c. of the serum from a case (during the first day of the illness) into a healthy individual was followed about eight days later by an attack of the disease. A similar effect was produced with the serum after it had been passed through a Berkefeld filter—all the inoculation experiments being performed at a distance from the original location of the disease. The virus has also been found to pass a Chamberland F filter. The view was therefore put forward that the causal agent belonged to the category of the ultra-microscopic viruses. Owing to the prevalence of the disease during the summer season Doerr considered there was justification for the popular view that it was associated with the bite of the dipterous fly, *Phlebotomus pappatasii*. This was borne out by the fact that on feeding such flies on a sick person, transporting them to a locality free from the disease and allowing them to bite healthy individuals, the affection was reproduced. An apparently identical disease occurs in Malta, and was first investigated by Birt under the name of "Phlebotomus Fever." This observer fully confirmed Doerr's results, the condition again being reproduced by infected flies, which, however, were found not to manifest infectivity earlier than seven days after biting. This last fact appeared to indicate that the causal organism has a developmental cycle in the fly. Other phlebotomus flies are also able to transmit the disease.

The Royal Air Force Sandfly Fever Commission carried out extensive experiments on the infectivity of the sand-fly, infectivity being determined by the occurrence of the characteristic fever in volunteers who had been bitten by captive flies. It was found that in an endemic area all phlebotomi are not infected with the virus and that the infection of the insect is not hereditary, but the experiments indicated that the infection can be transmitted to insects in their breeding-grounds by the larvæ ingesting the dejecta or dead remains of the adult flies (Whittingham). It was also noted that atmospheric temperature influenced the infectivity of the flies; thus insects, proved

to be infective when the temperature was above 65° F., became non-infective after a fall below 60° F. This Commission also demonstrated the presence of spirochætes of the leptospira type in six cases in Malta (p. 700). Kligler and Ashner, however, failed to recover a leptospira from patients or to detect it in infected flies. The serum of recovered cases when mixed with the virus has an anti-infective action.

Poradenitis (Climatic Bubo or Lymphogranuloma Inguinale)

This disease, which is spread by sexual contact, appears in the male chiefly as a subacute inflammatory swelling of the inguinal lymph glands, which tend to suppurate and produce a chronic sinus. Preceding the glandular enlargement there is a small lesion at the site of inoculation on the penis, which soon heals. In the female the inguinal glands may be similarly affected, but if the primary lesion is situated posteriorly on the genitalia, the rectum and anus are affected, stricture resulting, and this together with chronic swelling and ulceration of the vulva constitutes "esthiomène," a condition which leads to persistent infectivity. Frei showed that in cases of this disease the skin reacted specifically to the intradermal injection of pus from a bubo diluted with saline and sterilised by heating at 60° C. An area of inflammation develops, which reaches its height in forty-eight hours; this reaction is of value in diagnosis, since a positive result is not obtained in other conditions. (The Frei reaction can also be obtained by injecting an emulsion of the brain of experimentally infected monkeys or mice as shown by D'Aunoy and others—*vide infra*.) Hellerström and Wassén transmitted the infection to monkeys by inoculation with material from affected glands. After intracerebral injection there was an incubation period of six to twelve days and then meningo-encephalitis developed which often proved fatal. Passages were successful and on transference to the human subject again the disease was reproduced (Levaditi). Mice are also susceptible to intracerebral inoculation. The virus is filterable, but it has little resistance to glycerol. Elementary bodies have been demonstrated by Miyagawa within histiocytes, polymorphs and glial cells. The size of the virus bodies is 0·125–0·175 μ.

Other Conditions which may be due to Viruses

Warts.—The infectious nature of the warts which are common in childhood has been established, and the fact that the virus is

filterable has been confirmed by Findlay and others ; also it survives the action of 50 per cent glycerol. A similar infectious agent has been proved to exist in specimens of *condyloma acuminatum* and probably in other papillomata, such as those of the larynx in children. In dogs and cattle infective warts are also met with According to Findlay's observations the virus of dogs' warts is not infective for man , further, an immune serum which neutralises the human virus has no effect on that of the dog. Bodies have been described both in the cytoplasm and the nuclei of cells in warts which have been regarded as similar to the inclusion bodies found in other lesions due to viruses. The transmissible papilloma of cottontail rabbits when transferred to domestic rabbits causes papillomata which frequently become epitheliomatous (Rous and Beard)

Molluscum contagiosum contains a filterable virus which reproduces the lesion in man but not in animals. Characteristic large acidophile inclusion bodies are found in considerable numbers in the epithelial cells of the lesion—designated molluscum bodies. The molluscum bodies contain minute granules (Lipschutz) similar to those demonstrable in fowlpox.

Trachoma.—The infective character of trachoma appears to be definitely established, but the nature of the causal agent has not yet been determined. Halberstädter and Prowazek described in trachoma cases inclusion bodies in the cytoplasm of the conjunctival epithelium, which at their earliest stage appeared as minute coccal forms stained blue with Giemsa's solution ; then red granules appeared in the middle of these, and finally only small eosinophile granules and rods, about 0·25 μ in length, were seen. There is still doubt as to the nature of such bodies ; according to Bengtson, they may result from the action of lysozyme (p. 255) on bacteria in the conjunctival sac. Transmission of the disease to monkeys by filtrates of material from cases has been claimed. Noguchi has isolated from the lesions of trachoma a small Gram-negative motile bacillus with a single terminal flagellum, *B granulosis*, which grows well aerobically at 30° C. on agar containing defibrinated or citrated horse blood or serum. He claimed to have produced the disease in monkeys by injecting pure cultures of this organism beneath the conjunctiva, but this has not been confirmed ; further, the organism does not appear to be invariably present in trachoma lesions (McCartney and Mayou ; Bengtson). Olitsky and others have isolated a somewhat similar organism, *B. simiæ*, from spontaneous conjunctivitis in monkeys. It grows on ordinary agar and inoculation with pure cultures has reproduced the disease in these animals.

Inclusion Conjunctivitis.—A form of mild, persistent conjunctivitis occurring a few days after birth, but found also in adults (" swimming bath conjunctivitis "), is characterised by inclusion bodies in the epithelial cells which resemble those in trachoma, although clinically the diseases are quite distinct. Similar inclusion bodies have been found in epithelial cells from the cervix uteri and the male urethra of adults. The disease has been reproduced in man and baboons by inoculation with sterile filtrates of secretion from cases at the acute stage. Previous inoculation of the baboon's conjunctiva with trachoma does not confer immunity to inclusion conjunctivitis (Thygeson).

CHAPTER XXVI

PATHOGENIC PROTOZOA: MALARIA PLASMODIA; ENTAMŒBA HISTOLYTICA OF TROPICAL DYSENTERY

MALARIA PLASMODIA

MALARIA (French, paludisme) is the most prevalent infection of man, being specially frequent in tropical and subtropical countries. It was identified in antiquity by the regularly recurring febrile attacks which characterise certain forms of the disease, and the specific effect of quinine in abolishing the fever afforded a therapeutic method of diagnosis. The action of malaria in producing a great variety of diseased manifestations and in conducing to racial degeneration has also been recognised. It has now been long established that the malarial fevers are protozoal infections, there being different forms of the parasite. These belong to the *Hæmosporidia* (an order of the *Sporozoa*), which are blood parasites, infecting the red corpuscles of mammals, reptiles, and birds. The parasite is generally known by the generic name *Plasmodium*, and three well-defined species have been recognised: *Plasmodium vivax* of Benign tertian malaria, *Plasmodium malariæ* of Quartan malaria, and *Plasmodium falciparum* (or *Laverania malariæ*) of Malignant tertian malaria. The parasite was first observed by Laveran in 1880, and his discovery received confirmation from the independent researches of Marchiafava and Celli, and later from the researches of many others in various parts of the world. Golgi supplied important additional information, especially in relation to the sporulation of the organism and the varieties in different types of malarial fever. Valuable work on the subject was done by Manson, and to him specially belongs the credit of regarding the exflagellation of the organism as a preparation for an extra-corporeal phase of existence. By induction he arrived at the belief that the cycle of existence outside the human body probably took place in the mosquito. It was specially in order to discover, if possible, the parasite in this insect, that Ross commenced his long series of observations, which were ultimately crowned with success.

After patient and persistent search, he found rounded pigmented bodies in the wall of the stomach of a dapple-winged mosquito (a species of *Anopheles*) which had been fed on the blood of a malarial patient. The pigment in these bodies was exactly similar to that in the malaria parasite, and he excluded the possibility of their representing anything else than a stage in the life-cycle of the organism. He confirmed this discovery and obtained corresponding results in the case of the *Proteosoma* (*Hæmoproteus*) infection of birds, where the parasite is closely related to that of malaria. He was able to trace all stages of the development of this organism from the time it entered the stomach of the insect along with the blood from infected birds, till the time when it settled in a special form in the salivary glands. Ross's results were published in 1898. Exactly corresponding stages were afterwards found in the case of the different species of the human parasite, by Grassi, Bignami and Bastianelli; and these with other Italian observers also supplied important information regarding the transmission of the disease by infected mosquitoes. Abundant additional observations, with confirmatory results, were supplied by Koch, Daniels, Christophers, Stephens and others. Wherever malaria has been studied the result has been the same. Lastly, we may mention the striking experiment carried out by Manson by means of mosquitoes fed on the blood of patients in Italy suffering from mild tertian fever. The insects, after being thus fed, were taken to London, and allowed to bite human subjects, Manson's son, Dr. P. Thurburn Manson, and Mr. George Warren offering themselves for the purpose. Neither of them had been abroad or previously exposed to malaria infection. The result was that infection occurred; the parasites appeared in the blood, and were associated with an attack of tertian fever. Ross's discovery has not only been a means of elucidating the mode of infection, but, as will be shown below, has also supplied the means of successfully combating the disease.

From the zoological point of view the mosquito is regarded as the definitive host of the parasite, the human subject as the intermediate host. But in describing the life-history, it will be convenient to consider, first, the cycle in the human body, and, secondly, that in the mosquito. We shall first give a general account of the life-history and then describe the features of the different species.

The Asexual Cycle in the Human Subject—Schizogony.—With regard to this cycle (Plate V., Fig. 22, *a–j*), it may be stated that the parasite is conveyed by the bite of the mosquito

in the form of a small filamentous cell—*sporozoite,* which penetrates a red corpuscle and becomes a small amœboid organism or *trophozoite.* There is then a regularly repeated asexual cycle of the parasite in the blood, the length of which cycle determines the type of the fever. During this cycle there is a growth of the trophozoites within the red corpuscles up to their complete development; schizogony then occurs. The onset of the febrile attack corresponds with the stage of schizogony and the setting free of the *merozoites, i.e.* with the production of a fresh generation of parasites. These soon become attached to, and penetrate into the interior of, the red corpuscles, becoming intra-corpuscular trophozoites; the cycle is thus completed. The parasites are most numerous in the blood during the development of the pyrexia, and, further, they are also much more abundant in the capillaries of internal organs than in the peripheral blood; in the malignant type, for example, the process of schizogony is practically confined to the former and this is associated with greater clinical severity.

In addition to these forms which are part of the ordinary asexual cycle, there are derived from the trophozoites other forms, called *gametocytes,* or sexual cells, which tend to be produced especially when the infection has lasted for some time. These remain unaltered during successive attacks of pyrexia, and undergo no further change until the blood is removed from the human body. In the simple tertian (*P. vivax*) and quartan (*P. malariæ*) fevers (*vide infra*) the gametocytes are rounded in form, resembling somewhat in appearance the fully developed trophozoites before schizogony, whereas in the malignant type (*P. falciparum*) they have a characteristic crescent-like, or sausage-shaped form; hence they are often spoken of as "crescents."

The various forms of the parasite seen in the human blood may now be described more in detail.

1. *The Merozoites* are the youngest and smallest forms resulting from the segmentation of the adult amœbula or *schizont.* They are of round or oval shape and of small size, usually not exceeding 2 μ in diameter; the size, however, varies somewhat in the different species. A nucleus and peripheral protoplasm can be distinguished (Fig. 174, *h*). The former appears as a small rounded body which usually remains unstained, but contains a minute mass of chromatin which stains a deep red with the Romanowsky method, the peripheral protoplasm being coloured fairly deeply with methylene blue. The merozoites show little or no amœboid movement; at first free in the plasma, they

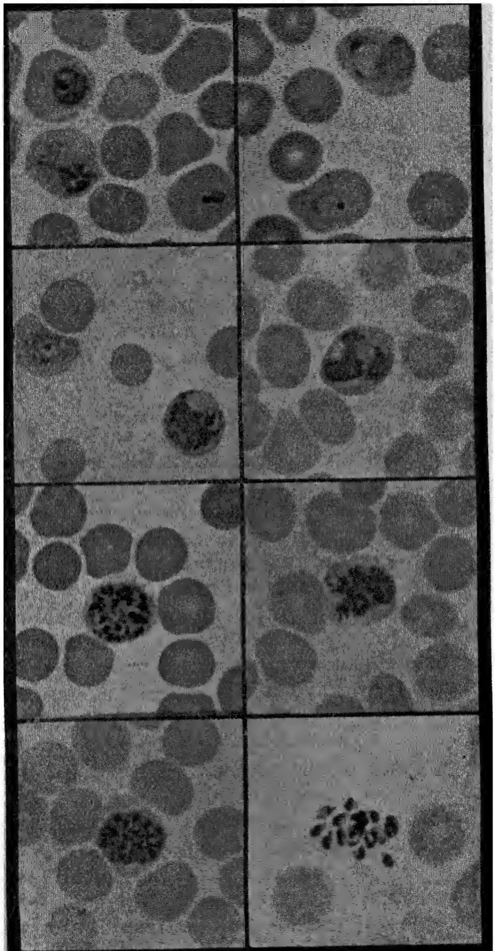


Fig. 174.—Various phases of the benign tertian parasite (*Plasmodium vivax*) *a*, *b*, trophozoites; *c*, *d*, developing schizonts; *e*, *f*, *g*, segmenting schizonts; *h*, free merozoites. ×1000.

soon attack the red corpuscles, within which they become the trophozoites. If the blood, say in a mild tertian case, be examined in the early stages of pyrexia, one often finds at the same time schizonts, free merozoites, and the young trophozoites within the red corpuscles.

2. *Trophozoites.*—These are the parasites which have invaded the red corpuscles (Fig. 174 *a*, *b*). They usually occur singly in the red corpuscles, but sometimes two or more may be present together. As seen in fresh blood, the youngest or smallest forms are minute colourless bodies, of about the same size as the merozoites; they exhibit more or less active amœboid movement, showing marked variations in shape. The amount and character of the amœboid movement varies somewhat in different species. As they increase in size, pigment appears in their interior as minute dark brown or black specks, and gradually becomes more abundant (Plate V., Fig. 22 *c*, *d*, *e*). This pigment is elaborated from the hæmoglobin of the red corpuscles, the parasite growing at the expense of the latter. The red corpuscles thus invaded may remain unaltered in appearance (*P. malariæ*, Fig. 175), or may become swollen and pale (*P. vivax*, Fig. 174). In stained specimens a nucleus may be seen in the parasite as a single concentrated mass of chromatin or as several separated chromatin granules, coloured a deep red by the Romanowsky stain. The protoplasm of the parasite, which is coloured of varying depth of tint with methylene-blue, shows great variation in configuration. The young parasites not infrequently present a "ring-form" due to the formation of a large vacuole, the nucleus being displaced to one side (Figs. 174 *b*, 175 *b*). These ring-forms are met with in all the varieties of the parasite, but they are especially common in the case of the malignant parasite, where they are of smaller size and of more symmetrical form than in the others.

Within the red corpuscles the parasites gradually increase in size till the mature form (*schizont*) is reached (Fig. 174 *c*, *d*). In this stage the parasite loses its amœboid movement more or less completely, has a somewhat rounded form, and contains a considerable amount of pigment. In the malignant form it only occupies a fraction of the red corpuscle (Fig. 176 *b*). The adult parasites may then undergo schizogony, but not all of them do so; some become degenerated and ultimately break down.

3. *Schizonts.*—In the process of schizogony the nuclear outline becomes lost, and the chromatin becomes divided into a number

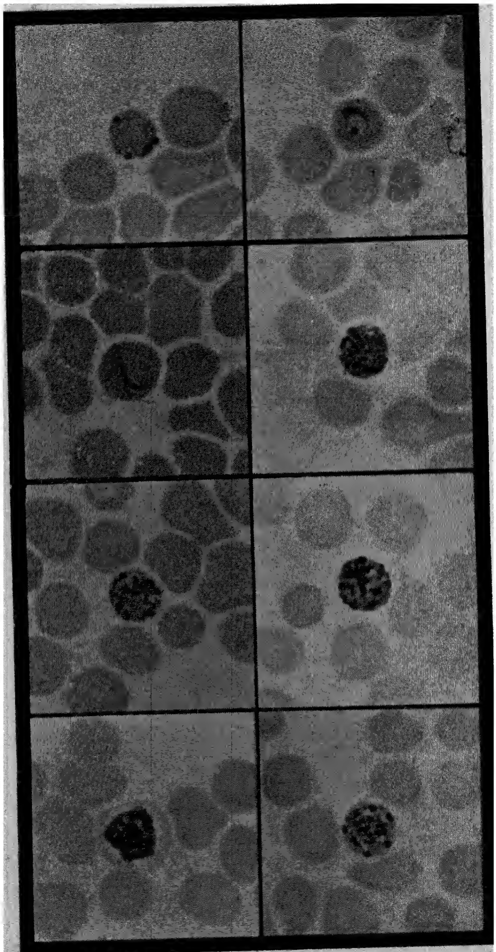


FIG. 175.—Various phases of the quartan parasite (*Plasmodium malariæ*). *a*, trophozoite showing marginal infection; *b*, trophozoite; *c*, trophozoite (*quartan band*); *d*, schizont; *e*, *f*, *g*, *h*, segmenting schizonts. ×1000.

of small granules which are scattered through the protoplasm ; the latter then undergoes corresponding segmentation and the small merozoites result. The pigment during the process becomes aggregated in the centre and is surrounded by a small quantity of residual protoplasm. (Schaudinn stated in the case of *P. vivax* that schizogony begins by a sort of primitive mitosis, which is then followed by simple multiple fission.) The merozoites are of rounded or oval shape, as above described, and are set free by the rupture of the envelope of the red corpuscles. The pigment also becomes free and may be taken up by leucocytes, the merozoites being free from pigment. The number and arrangement of the merozoites within the schizont vary in the different types. In *P. malariæ* there are 6–12, and the segmentation is in a radiate manner giving rise to the characteristic daisy-head appearance (Fig. 175, *g*) ; in *P. vivax* they number 15–20, and have a somewhat rosette-like arrangement (Fig. 174, *g*) ; in *P. falciparum* there are usually 6–24 merozoites of small size and somewhat irregularly arranged (Fig. 176, *c*).

Gametocytes.—These are sexual cells which are formed from certain of the trophozoites, and which undergo no further development in the human subject. In the mild tertian and quartan fevers they are rounded and resemble somewhat the largest trophozoites (Figs. 177, 178). The female cells, *macrogametocytes*, are relatively of large size, measuring 12–14 μ in diameter ; they contain coarse grains of pigment, and the protoplasm stains somewhat deeply blue with the eosin-methylene-blue stains, while the nucleus is small, compact, deeply staining, and is commonly situated near the margin of the parasite. The male cells, *microgametocytes*, are smaller, and the protoplasm stains faintly ; the nucleus, generally in the centre, is large, somewhat diffuse, and often forms a broad band or spindle stretching across the cell. In first infections with *P. vivax* gametocytes appear in the peripheral blood on the seventh day of fever, and mosquitoes which ingest the blood on about the tenth day may become infected (James). In *P. falciparum* the gametocytes have the special crescentic or sausage-shaped form. They measure 9–14 by 2–3 μ, and occasionally a fine curved line is seen joining the extremities on the concave aspect, which represents the envelope of the red corpuscle (Fig. 176 *d, e*). They are colourless and transparent, and are enclosed by a distinct membrane ; in the central part there is a collection of pigment and granules of chromatin. The male crescents (Plate V., Fig. 23 *f*, Fig. 176 *d*), can be

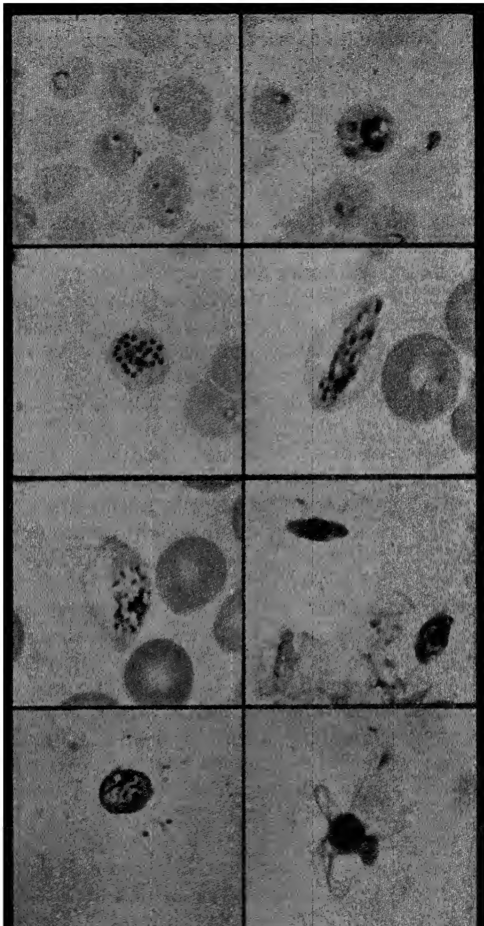


FIG. 176.—Various phases of the malignant malaria parasite (*Plasmodium falciparum*).

a, trophozoites in peripheral blood.
b, schizont (smear from liver).
c, segmenting schizont (smear from liver)
d, microgametocyte (male crescent).
e, macrogametocyte (female crescent).
f, *g*, *h*, changes in form undergone by the crescents outside the body —— *h*, microgametocyte showing exflagellation.
a–c and *f–h* × 1000
d and *e* × 1200
(Figures *f*, *g* and *h* are from preparations lent by the late Sir Patrick Manson.)

distinguished from the female (Fig. 176 *e*) by their appearance; the former are somewhat sausage-shaped, the pigment is less dark and more scattered through the cell, and there are several granules of chromatin; the latter have more pointed ends and their substance stains more deeply with the blue, the pigment is dark and concentrated, often in a small ring, and there are one or two masses of chromatin in the centre of the crescent. According to the Italian observers, the early forms of the crescents are somewhat fusiform in shape and are produced in the bone-marrow. The fully developed crescents do not appear in the blood till several days after the onset of the fever, and they may be found a considerable time after the disappearance of the pyrexial attacks; they are most numerous in cases in which the infection has lasted for some time. They are also little, if at all, influenced by the administration of quinine. Ross and Thomson enumerated directly (p. 809) the malaria parasites in the blood at different stages of the disease, and found that a certain relationship exists between the asexual and the sexual forms, a rise in the number of the former being followed eight to ten days later by a rise in the number of the latter; they accordingly considered that this is probably the period necessary for the development of the sexual forms. They considered also that the long persistence of crescents in the blood after the fever has ceased, is due not to the long survival of individual crescents, but to their being constantly replenished from asexual forms which persist in the blood and pass through the ordinary process of schizogony, fever only occurring when the number of asexual forms reaches some hundreds per cubic millimetre.

Relapses and Recurrences.—It is well known that after a patient has apparently recovered from malarial fever a relapse or recurrence may take place without fresh infection, sometimes many years afterward, and Schaudinn claimed that the macrogametocyte of tertian fever may by a process of parthenogenesis give rise to merozoites, which in their turn infect the red corpuscles and start the cycle again. The observations of Ross and Thomson, just referred to, support the view of Bignami and others, that recurrences depend on the survival of asexual forms in small numbers, which pass through the ordinary cycle and only produce fever when they again become sufficiently numerous. It is possible also, that malaria parasites may penetrate into endothelial cells and remain latent or even develop there; a recurrence would then coincide with the setting free subsequently of these organisms in the blood

stream. Thus, James has shown by experiments in fowls that an avian malaria parasite may undergo a schizogenous cycle in reticulo-endothelial cells. Certain unusual appearances of the parasites met with in chronic infections have been interpreted as special asexual resistant forms which may survive in the blood during the latent periods of the disease.

The Sexual Cycle in the Mosquito—Sporogony.—As already explained, this starts from the gametocytes. After the blood is shed, or after it is swallowed by the mosquito, two important phenomena occur, namely, (*a*) the full development of the sexual cells or gametocytes, and (*b*) the fertilisation of the female cell (Plate V., Fig. 22 *m–q*). If the blood from a case of malignant infection be examined in a moist chamber, preferably on a warm

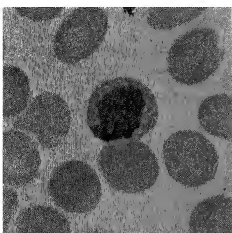


FIG. 177.—Gametocyte of *Plasmodium vivax*. ×1000.

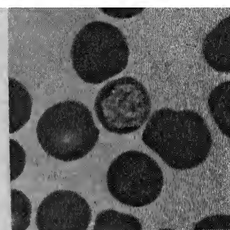


FIG. 178.—Gametocyte of *Plasmodium malariæ*. ×1000.

stage, under the microscope, both male and female gametocytes may be seen to become oval and afterwards rounded in shape (Fig. 176 *f*, *g*). Maturation then takes place by the extrusion of part of the nuclear chromatin, this process corresponding to the formation of polar bodies. Thereafter, in the case of the male cell, a vibratile or dancing movement of the pigment granules can be seen in the interior, and soon several flagellum-like structures shoot out from the periphery (Fig. 176 *h*). They are of considerable length but of great fineness, and often show a somewhat bulbous extremity. By the Romanowsky method they have been found to contain a delicate filament of chromatin. They represent the male cells proper, and are known as *microgametes*. They become detached from the sphere and move away in the surrounding fluid. Fertilisation (syngamy) occurs by the fusion of a microgamete with the mature female cell (*macrogamete*), the chromatin of the two cells afterwards forming

a single nucleus. Similar changes occur in the gametocytes of the other species, but, as has been said, the cells are rounded from the first. The fertilised female cell is now generally spoken of as a *zygote* or *oökinete.*

It has been established that the phenomena just described occur within the stomach of the mosquito, and that the fertilised cell or zygote which becomes elongated and motile, penetrates the stomach wall and settles between the muscle fibres ; on the second day after the mosquito has ingested the infected blood, small rounded cells about 6 to 8 μ in diameter, and containing clumps of pigment, may be found in this position. (It was, in fact, the character of the pigment which led Ross to believe that he had before him a stage in the development of the malaria parasite.) A distinct membrane called a *sporocyst* forms around the zygote, and on subsequent days a great increase in size takes place, the cysts coming to project from the surface of the stomach into the body cavity. The zygote divides into a number of cells called *sporoblasts,* and these again divide and form a large number of filiform cells which have a radiate arrangement ; these were called by Ross "germinal rods," but are now usually known as *sporozoites.* The full development (*sporogony*) within the sporocyst occupies over seven days, the length of time depending on the temperature. When fully developed the cyst measures about 60 μ in diameter, and appears packed with sporozoites. It then bursts, and the latter are set free in the body cavity. A number settle within the large veneno-salivary gland of the insect, and are thus in a position to be injected along with its secretion into the human subject. According to James, the sporozoites, to produce a successful infection, must be lying free in the common salivary duct of the mosquito at the time of biting. The sporozoites enter red corpuscles and become trophozoites, as above described. Daniels found that, in the case of *P. falciparum,* an interval of twelve days at least intervened between the time of feeding the mosquito and the appearance of the sporozoites in the gland. In the case of *P. vivax* in mosquitoes kept at 24° C. in a saturated atmosphere, the shortest interval was ten days (James). For the occurrence of the cycle in the mosquito a sufficiently high temperature and degree of humidity of the atmosphere are both essential. There is no evidence that the malarial parasites in the mosquitoes pass to the next generation in the eggs. An infected mosquito may rid itself of sporozoites by repeated biting but, apart from this, when once infected it remains infective for long periods, *e.g.* three months in the case of the benign tertian parasite ;

also the infection may persist over the winter when the insect hibernates (James).

It will thus be seen that in the human subject the parasite passes through an indefinite number of regularly recurring asexual cycles, with production of collateral sexual cells, and that in the mosquito there is one cycle which may be said to start with the fertilisation of the female gamete.

Varieties of the Malaria Parasites.—The view originally propounded by Laveran was that there is only one species of malaria parasite, which is polymorphous, and presents slight differences in structural character in the different types of fever. It may, however, be accepted that there are at least three distinct species which infect the human subject. Practically all are agreed as to a division into two groups, one of which embraces the parasites of the milder fevers—" winter-spring " fevers of Italian writers—there being in this group two distinct species, for the quartan and tertian types respectively ; whilst the other includes the parasite of the severer forms—" æstivo-autumnal " fevers, malignant or pernicious fevers of the tropics, or irregularly remittent fevers. Formerly Italian writers distinguished several varieties of the latter parasite, though the morphological differences described were slight. Further observations, however, threw doubt on this distinction, and the evidence tended to show that there is a single species. Opinion also varied as to the cycle of this parasite : according to some observers, twenty-four hours, according to others forty-eight hours ; though there is more evidence in support of the latter view, and the term " malignant tertian " or " sub-tertian " is frequently used. The fever is often of an irregular type and multiple infection is probably common. We may therefore speak of three well-defined species of the malaria parasite of man : *Plasmodium vivax* of benign tertian malaria, *Plasmodium malariæ* of quartan malaria, and *Plasmodium falciparum* (*Laverania malariæ*) of malignant or sub-tertian malaria.

We shall now give the chief distinctive characters of these :

1. *The Parasite of Benign Tertian Fever* (*P. vivax*).—The cycle of development is completed in forty-eight hours. The trophozoites have a less refractile margin than in the quartan type, and are thus less easily distinguished in the fresh blood ; the amœboid movements are, however, much more active, while longer and more slender processes are given off. The infected corpuscles become enlarged and pale, and may show deeply stained points by the Romanowsky method—" Schüffner's dots." The pigment within the parasite is fine and of yellowish-

brown tint. The mature schizont is rather larger than in the quartan, has a rosette appearance, and gives rise to from fifteen to twenty merozoites, though sometimes even more occur; these have a somewhat oval shape (Fig. 174 *g*, *h*).

When the blood is examined parasites of the benign tertian variety may be found simultaneously in several stages of development, and in such cases the fever is of a quotidian type. It was believed that this pointed to a double infection, but the observations of James indicate another possible explanation. Patients were investigated whose primary attack of malaria was caused by a single bite of one mosquito infected with a pure strain of *P. vivax* under experimental conditions. It was found that the "initial" stage of infection, lasting two to five days, was characterised by gradually increasing irregular fever without rigors. This was followed in 80 per cent. of cases by the "developed" stage with quotidian fever and rigors, which often lasted more than ten days. The "terminal" stage then set in, the quotidian fever changing to that of tertian type and the severity of the paroxysms gradually diminishing. Examination of the peripheral blood throughout these stages showed that soon after the first appearance of the parasites different phases of growth are found together—an occurrence which is explained by sporozoites received from the mosquito not being all equal in their rate of development. Then in the developed stage two groups become dominant and determine the quotidian fever. Finally, the tertian type of attack is due to impending recovery, one of the two groups of parasites having disappeared before the other.

2. *Parasite of Quartan Fever* (*P. malariæ*).—The cycle of development in man is seventy-two hours, and produces pyrexia every third day; double or triple infection may, however, occur. In fresh specimens of blood the outline is more distinct than that of the tertian parasite, and amœboid movement is less marked. Only the smaller forms show movement, and this is not of active character. The infected red corpuscles do not become altered in size or general appearance but by prolonged staining a stippling ("Ziemann's stippling") may be demonstrated. The pigment within the parasite is in the form of coarse granules, of dark brown or almost black colour. Medium-sized parasites may appear as band-shaped forms in the corpuscles (Fig. 175 *c*). The fully developed schizont has a "daisy-head" appearance, dividing by regular radial segmentation into from six to twelve merozoites, which, on becoming free, are rounded in form (Fig. 175 *e*, *f*, *g*, *h*).

In both the quartan and tertian fevers all the stages of development can be readily observed in the peripheral blood The gametocytes have a rounded form as described above.

It may be noted here that the quartan type of infection may persist for a longer period than benign tertian malaria.

3. *The Parasite of Malignant or Sub-Tertian Fever* (*P. falciparum*, or *Laverania malariæ*).—The cycle in the human subject probably occupies forty-eight hours, though this cannot be definitely stated to be always the case (*vide supra*). The trophozoites in the red corpuscles are of small size, and their amœboid movements are very active; they often, however, pass into the quiescent ring form (Fig. 176 *a*). The pigment granules, even in the larger forms, are few in number and very fine. The infected red corpuscles may be unaltered or tend to shrivel and assume a deeper or coppery tint, sometimes they are swollen and decolorised. They may contain "Maurer's dots," which resemble those of Schüffner, but are scantier, larger, and more irregular. The young forms are often found at the edge of the red cells and are elongated ovals or streaks; the rings frequently project from the margin of the corpuscle. The fully developed schizont usually occupies less than half the red corpuscle, and produces from six to twenty-four merozoites, somewhat irregularly arranged and of minute size (Fig. 176 *c*). Schizogony takes place almost exclusively in the internal organs, spleen, etc. Usually no schizonts can be found in the blood taken in the usual way, but they may be observed in the very severe or pernicious type of the disease. The proportion of infected red corpuscles is also much larger in the internal organs. The gametocytes have the crescentic form, as already described.

Cases of infection with the malignant parasite sometimes assume a pernicious character, and then the number of organisms in the interior of the body may be enormous. In certain fatal cases with coma the cerebral capillaries appear to be almost filled with them, many parasites being in process of schizogony; and in so-called algid cases, characterised by great collapse, a similar condition has been found in the capillaries of the omentum and intestines. The process of blood destruction, present in all malarial fevers, reaches its maximum in the malignant class, and the brown or black pigment elaborated by the parasites—in part after being taken up by leucocytes, chiefly of the mononuclear class—becomes deposited in various organs, spleen, liver, brain, etc., especially in the endothelium of vessels and the perivascular lymphatics. In the severer forms also brownish-yellow pigment is apparently derived from liberated hæmoglobin, and accumulates in various parts, especially in the liver cells: most of this latter gives the reaction of hæmosiderin.

Plasmodium ovale.—Attention has been drawn in recent years to a form of malaria parasite which probably constitutes a fourth species. It has been reported by Stephens in cases of African

malaria and designated *P. ovale*. The fever is of the tertian type but in blood films parasites are found which show some similarity to *P. malariæ* (of quartan malaria) though the pigment is lighter in colour and less coarse. The red cells tend to be enlarged (as in *P. vivax* infection) and often exhibit frayed or fimbriated edges. Medium-sized trophozoites may assume an oval shape and the red cell may also be oval. A pronounced feature of the blood corpuscles is their granular stippling likened to the Schüffner's dots of benign tertian malaria, but the granules are more distinct, coarser, and stain more intensely. The schizont yields 6 to 12 merozoites. The sexual cycle of this parasite has been studied in *Anopheles maculipennis*. Blood films are best stained by the technique of Shute as described by James. The infection produced experimentally in general paralysis is very mild and becomes cured spontaneously.

Cultivation.—Bass and Johns succeeded in obtaining growths of the parasites of tertian and malignant fevers outside the body. The first cultures were obtained in defibrinated blood from malarial patients, to which was added 1 per cent. of a 50 per cent. solution of dextrose in water. Growth of the parasites took place within the red corpuscles, but only under anaerobic conditions, so that a layer of serum at least half an inch in depth above the sedimented corpuscles was necessary. Under such circumstances, the parasites underwent enlargement and afterwards passed through the stage of schizogony. The merozoites after becoming free are destroyed by leucocytes, but if measures are taken to prevent the presence of these, other generations of growth may be obtained in similarly prepared tubes of blood with sufficient serum. The parasites flourish only in the superficial layers of the sedimented corpuscles, and the most suitable temperature is 37°–41° C. Cultivation of the malaria organisms has since been effected by Thomson and McLellan and others. According to J. G. and D. Thomson, removal of the leucocytes is unnecessary. These observers found that in cultures the malignant parasites showed a tendency to clump before and during the stage of sporulation ; on the other hand the benign tertian parasites did not clump.

Knowles recommends the following method for the cultivation of malaria parasites as an aid in diagnosis. Five c.c. of blood are withdrawn from a vein with a sterile syringe, the interior of which has been carefully washed with saline. The blood is then added to a sterile stoppered flask containing glass beads and defibrinated Sterile stoppered tubes $12\frac{1}{2} \times 1\frac{1}{2}$ cm. are used for the cultures and into each is pipetted a drop of 50 per cent. aqueous solution of the *purest* dextrose which has been sterilised by the intermittent method and to this is added defibrinated blood to a depth of $2\frac{1}{2}$ cm. The upper part of the tube is warmed and a rubber teat is immediately fitted to the mouth ; this produces partial anaerobiosis. The

cultures are incubated at 37° C. and examined after twelve hours and if necessary after twenty-four and forty-eight hours. This is done by aspirating with a capillary pipette some of the upper layer of the sedimented red cells and making films which are stained in the usual way.

General Considerations.—The development of the malaria parasites in the mosquito and infection of the human subject through the bites of this insect, have, by the work of Ross and others, as detailed above, become definitely established. These facts, moreover, point to several definite methods of prevention of infection, which have to a certain extent already been practically tested. The extensive observations carried out go to show that all the mosquitoes which act as hosts of the parasite belong to the genus *Anopheles*; of these there are a large number of species, and in several of them the parasite has been found. Some of these anopheles occur in Great Britain, especially in regions where malaria formerly prevailed. The opportunity for infection from cases of malaria returning from the tropics to this country thus exists, and such infection has occurred. The breeding places of the insects are chiefly, though not exclusively, in stagnant pools and other collections of standing water, and accordingly the removal, where practicable, by drainage of such collections in the vicinity of centres of population, the covering in of wells, etc., and the killing of the larvæ by petroleum sprinkled on the water, have constituted the most important measures in localised areas. This procedure has been carried out in various places with marked success. Also in more open waters fish have been used for the control of mosquitoes. On the other hand, where there are large populous areas, as in India, it has been found almost impracticable to carry out such measures efficiently. Another method is the protection against mosquito bites by netting, it being fortunately the habit of the anopheles rarely to become active before sundown. The experiments of Sambon and Low in the Campagna proved that individuals using these means of protection may live in a highly malarial district without becoming infected. The administration of antimalarial drugs to persons living in highly malarial regions, in order to *prevent* as well as to treat infection, has also been recommended. There appears to be general agreement that in India the properly controlled administration of such drugs (p. 918) must, in the meantime at least, be the chief means of combating the disease. In the tropics the natives in large proportion suffer from malarial infection, and one would accordingly expect that infection of the mosquitoes in the

neighbourhood of native settlements would be common. This has been found to be actually the case, and the dwellings of Europeans should as far as possible be at some distance from the native centres of population. So far as is known, none of the lower animals have been found to take the place of man as intermediate host to the parasites of malaria.

It may be mentioned, although not bearing on the natural modes of infection, that the disease can also be communicated from one person to another by injecting the blood containing the parasites. Usually ½ to 1 c.c. of blood is infective, but the result is more certain in intravenous than in subcutaneous injection. In such cases there is an incubation period, usually of from seven to fourteen days, after which the fever occurs; the same type of fever is reproduced as was present in the patient from whom the blood was taken. This method of inoculation, as well as that by the bites of infected mosquitoes, has been applied in recent years for the treatment of general paralysis, and it has been shown that malarial attacks artificially produced in this way exert a beneficial effect in this disease. The question has not yet been settled whether the therapeutic effect is due entirely to the high temperature produced.

Plasmodium knowlesi.—This parasite deserves mention here as it has been utilised in the malaria therapy of general paralysis of the insane. It occurs as a commensal in the blood and tissues of the Malayan monkey (*Silenus irus*) but when transmitted to *Silenus rhesus* and *Macacus rhesus* produces a severe and even fatal infection. Its pathogenicity on experimental inoculation in man has been demonstrated by Knowles and Gupta, and by van Rooyen and Pile. The latter workers have recorded successful therapeutic results with it in general paralysis. The infection can easily be maintained in *M. rhesus* monkeys in the laboratory and the human infection can be induced by intravenous or intramuscular injection of defibrinated blood from these animals. Moreover, such blood when kept at 0° C. retains its infectivity for the human subject for eight days and for sixteen days in the case of the monkeys. In man the incubation period is usually eight days. The resulting fever is of quotidian type. The infection can be immediately terminated when necessary by a single intramuscular injection of quinine (10 grains), though atebrin may be ineffective. The parasite resembles the other *Plasmodia* in general characters. Typical ring forms with one or two chromatin dots are a common feature of blood films. Band forms of the parasite in red cells (cf. *P. malariæ*) are frequently observed. The pigment granules of the parasite tend to be large and dark-coloured (Fig. 179). The red cells are not usually enlarged, but a fully developed parasite may destroy practically the whole erythrocyte. Schizonts divide irregularly into 12 or 13 merozoites. The gametocytes are rounded in shape like those of *P. vivax* and *P. malariæ*.

The Pathology of Malaria.—While much work has been done on the malarial parasite, relatively less attention has been directed to the processes by which it produces its pathogenic effects. It may be said that the organisms are not always equally prevalent in the circulating blood, and at certain stages tend to be confined in the internal organs. Some of the pathogenic effects are associated with particular stages in the life-cycle. Thus the pyrexia occurs when the stage of schizogony is actively in progress, but nothing definite is known as to its real nature or mode of production. We can better explain the anæmia which is so pronounced in cases where the disease is of long standing, and which is due to the actual destruction of red blood corpuscles. The parasite in its sojourn in these cells absorbs their pigment and thus destroys their function; this is further indicated by the activity displayed by the red marrow in its attempts to make good the loss sustained by the blood. One of the most interesting events in malaria, and one that links it with bacterial infections, is the reaction of the leucocytes. It has been shown that during the apyrexial stages the total number of leucocytes varies greatly, but that there is always an increase of the mononuclear cells, these frequently numbering 20 per cent. or more of the whole, and sometimes even outnumbering the polymorphs. This is such an important feature that in cases where the parasites themselves cannot be demonstrated in the blood, the mononuclear reaction along with the presence of pigment in the mononuclear cells (due to phagocytosis of pigmented parasites

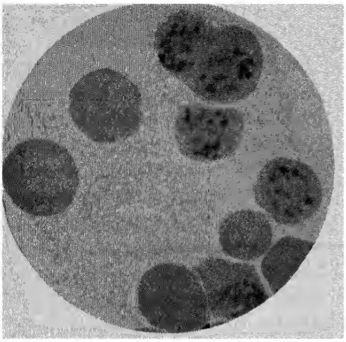


FIG. 179. — *Plasmodium knowlesi* (*monkey malaria parasite*) in a film of blood from a case of general paralysis under malaria therapy. Fully grown trophozoites in corpuscles which are of normal size. Note the coarse granules. Leishman's stain. ×1122. (From a photograph by Dr. C. E. van Rooyen).

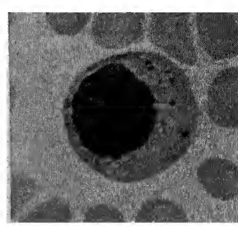


FIG. 180. — Mononuclear leucocyte from blood in a case of malaria (*quartan*) showing ingested particles of pigment. Leishman's stain. ×1000.

and pigment liberated after schizogony) has been taken as evidence that the case is really one of malaria (Fig. 180). The mononuclear reaction is specially interesting from the fact that in other protozoal diseases an activity of the same elements has been observed. Enlargement of the spleen is a marked feature, especially in chronic malaria; its prevalence in native children affords a rough indication of the incidence of the infection in regions where the disease is endemic.

The question of immunity to malaria is one of great importance. With regard to Europeans developing immunity, it is difficult to speak. In such a malaria-stricken region as the West Coast of Africa, the death-rate in residents of more than four years' standing is less than in the previous years, but this may be due to the survival of the more resistant immigrants. But there can be little doubt that malaria in the negro is a much less serious condition than in the European. Koch from his observations in New Guinea attributed this to the infection of the native children leading to the development of immunity in the adult community. He found, what had been independently noted by Stephens and Christophers in West Africa, that the greater number of the children harboured malarial parasites in their blood. The widespread presence of parasites in children might appear to preclude the possibility that the immunity of the adult is due to survival of the most resistant, but the infant mortality in these regions may be very high, and such a survival may be the real explanation. On the other hand, Koch stated that while an immunity appears to exist in native adults in malarial districts, this is only true of those born in the locality—natives coming from neighbouring non-malarial districts into the malarial region being liable to contract the disease. But of the existence of acquired immunity there can be no doubt, since a person who has previously suffered from malarial attacks may, while apparently in good health, harbour large numbers of parasites in the blood. It would appear that for the maintenance of immunity frequently repeated re-infection (superinfection) is required. At present, however, the facts available do not enable us to determine the relative parts played by the development of acquired immunity on the one hand, and the existence of a natural immunity on the other, in relation to insusceptibility to malaria. While a malarial infection may spontaneously undergo cure, the number of parasites diminishing until they are no longer detectable in the blood, nevertheless no form of antibody can be demonstrated. Some individuals are naturally resistant to malarial infection as tested by experimental inoculation (James).

Our knowledge regarding the relationship of blackwater fever to malaria is also in an unsatisfactory condition. Blackwater fever occurs, especially in Europeans, in tropical countries where malaria exists, and particularly after the malignant type contracted in West Africa. It is characterised by pyrexia, jaundice darkly coloured urine—the colour being due to altered hæmoglobin pigment—delirium and collapse, frequently ending in coma and death. By some the condition has been looked on as a separate disease, by others as the terminal stage of a severe malaria. With regard to the former view, no special parasite has yet been definitely demonstrated. Stephens has summed up the evidence for the second view by saying that malaria, apart from the occurrence of blackwater fever, is a relatively non-fatal disease ; that in the great majority of cases there is direct or indirect evidence of the subject of the condition having suffered from repeated attacks of malaria ; that while in all cases there must be an agent at work causing hæmolysis, the evidence in some cases points to the possibility of that agent being quinine. Christophers and Bentley came to the conclusion that the essential feature in blackwater fever is an extracellular destruction of red corpuscles in the blood plasma, a lysæmia as they call it. and that this is not directly due to parasitic, osmotic, or chemical actions, but to a specific hæmolysin arising in the body as the result of the repeated blood destruction. But the presence of such lysin in the serum has not been established. The occurrence of lysæmia may be precipitated by an acute attack of malaria especially when under certain circumstances this is associated with the administration of quinine. With regard to this view, however, it still remains to be determined what factors are responsible for the onset of an attack of blackwater fever. The evidence obtained by J. G. Thomson in investigations of the condition in Rhodesia has been strongly in support of the view that blackwater fever is the sequel of repeated attacks of malignant malaria. Fairley and Bromfield as a result of their observations have recently put forward the hypothesis that the hæmolytic substance of blackwater fever arises in chronic malignant malaria from some cellular metabolic breakdown precipitated by quinine, chill, etc., and its action is to lyse the corpuscles and then to convert the liberated oxyhæmoglobin to methæmoglobin which can be demonstrated in the plasma.

Methods of Examination.—The parasites may be studied by examining the blood in the fresh condition, or by permanent preparations. In the former case, a slide and cover-glass having been thoroughly cleaned, a small drop of blood from the finger or lobe

of the ear is caught by the cover-glass, and allowed to spread out between it and the slide. It ought to be of such a size that only a thin layer is formed. A ring of vaseline is placed round the edge of the cover-glass to prevent evaporation. For satisfactory examination an immersion lens is to be preferred. The amœboid movements are visible at the ordinary room temperature, though they are more active on a warm stage With an Abbe condenser a small aperture of the diaphragm should be used.

For diagnosis, as well as for detailed study of the parasites, permanent preparations are required; these are best made by means of dried films, which are then fixed and stained by one of the Romanowsky methods, as described on page 129. When such stains are not available, the dried films should be fixed by one of the methods described on page 111, and then stained by methylene-blue or thionin. In examining for the presence of malaria parasites it is most important that quinine should not have been administered before blood films are made. It must be remembered that the parasites may be very scanty in the blood, especially in early infections and in cases which have been treated with quinine. Accordingly, several hundred fields should always be examined. In latent cases of malarial infection, reappearance of parasites in the blood may follow an injection of adrenaline or tuberculin.

Ross's "thick film process" may be used to aid the finding of scanty organisms. Here about as much blood as is used in a hæmoglobin determination (20 c mm.) is taken on a slide, and, being spread out only so much as to occupy the area of an ordinary cover-glass, is allowed to dry. The hæmoglobin is removed by treating with distilled water, and the preparation is then fixed with methyl alcohol and stained by one of the Romanowsky methods; the parasites can then be readily found. Ross and Thomson have modified the method for enumeration purposes. They take a definite small amount of blood, say 1 c.mm, and discharge it on a slide as one or more droplets, which are dried and treated as above. The whole blood is then carefully searched with an oil immersion lens with the aid of a movable stage, and the total number of parasites present is counted. A thick and a thin film side by side on the same slide may also prove valuable.

The cultural method (*vide supra*) may also be resorted to in difficult cases and where the species cannot be determined by examination of films.

Entamœba Histolytica of Tropical Dysentery

In a previous chapter it has been pointed out that the term "dysentery" has been applied to conditions of different etiology, and the relations of bacteria as causal agents have been discussed (*vide* p. 546). We shall consider here that variety of tropical dysentery which is due to an amœba, and hence often known as *amœbic dysentery.*

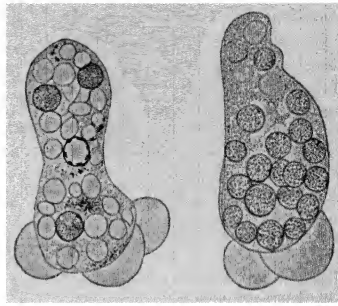
Among the early researches on the relation of organisms to dysentery probably the most important are those of Lösch, who noted the presence and described the characters of amœbæ in

the stools of a person suffering from the disease, and considered that they were probably the causal agents. Further observations on a more extended scale were made by Kartulis with confirmatory results, this observer finding the same organisms also in liver abscesses associated with dysentery. Councilman and Lafleur, working in Baltimore, showed that this variety of dysentery can be distinguished from other forms, not only by the presence of amœbæ, but also by its pathological anatomy. The intestinal lesions, to which reference is made below, are of a grave character, mortality is relatively high, and recovery, when it occurs, is protracted on account of the extensive tissue changes. The subject was, however, complicated by the fact that a somewhat similar organism—"*Amœba coli*"—had been previously found in the intestine in normal conditions and in other diseases than dysentery (by Cunningham and Lewis and others), and additional research confirmed these results. The characters of the common amœba of the colon and an amœba of dysentery were carefully worked out by Schaudinn, who recognised them to be quite distinct species, and gave to them the names of *Entamœba coli* and *Entamœba histolytica* respectively. Huber afterwards described an entamœba of dysentery, which in the encysted stage contained four nuclei. Viereck confirmed these observations and gave the name "*Entamœba tetragena*" to the organism. This organism was shown to have pathogenic properties. Further research has resulted in its being generally recognised that *E. histolytica* and *E. tetragena* are the same. Schaudinn's name of *E. histolytica* has been retained, but its process of encystment corresponds with that originally described in the case of *E. tetragena.* Moreover, a small entamœba, described by Elmassian under the name "*E. minuta*," is also now known to represent merely a stage in the life-history of *E. histolytica.* Within recent years several other species of intestinal amœbæ have been identified. These, like the *E. coli*, are non-pathogenic commensals (*vide infra*).

Entamœba histolytica, as seen in the stools of acute dysentery, occurs in the form of rounded, oval, or pear-shaped cells, the rounded cells measuring 15–50 μ, usually about 30 μ, in diameter (Figs. 181, 182, 183 and Plate VI., Fig. 24). When at rest, a somewhat clear, highly refractile ectoplasm and a granular or sometimes vacuolated endoplasm may sometimes be distinguished, though this is not always the case. The nucleus is rounded, as a rule about 7 μ in diameter, and is seen with difficulty; its position is usually excentric, and is sometimes quite at the margin of the endoplasm. In stained specimens it

is seen to be poor in chromatin, which is arranged as small granules under the nuclear membrane; a small chromatic karyosome is present in the centre of the nucleus (Fig. 183 A, B, C).

In perfectly fresh material very active amoboid movements are seen which are of a flowing type. When the activity of the organism lessens pseudopodia may be suddenly protruded and later retracted and these may consist almost entirely of ectoplasm. These appearances when present are characteristic and of great service in the identification of the organism. The amœboid movements may not lead to much change in position. Red corpuscles, remains of cells, and occasionally bacteria may be present within the amœbæ. The ingestion of red corpuscles, though not always present, is a characteristic feature of the organism, and thus of special importance in its recognition; a considerable number of red corpuscles may be contained in an amœba. The amœbæ usually die and undergo disintegration in a comparatively short time after being removed from the body; the stools ought therefore to be examined in *as fresh a state as possible*. According to Dobell *E. histolytica* does not ingest bacteria, and the presence of bacteria within the organism indicates an invasion of a degenerated amœba or, occasionally, a true parasitism by the bacteria.



a *b*

FIG. 181.—*Entamœba histolytica.*

a and *b*, amœbæ as seen in stools, showing blunt amœboid processes of ectoplasm. The endoplasm of *a* shows a nucleus, three red corpuscles, and numerous vacuoles; that of *b*, numerous red corpuscles and a few vacuoles.

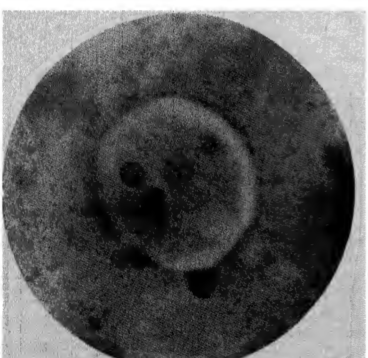


FIG. 182.—*Entamœba histolytica* in film from faeces. Stained iron hæmatoxylin. ×750.

Multiplication of the amœbæ occurs by division into two equal

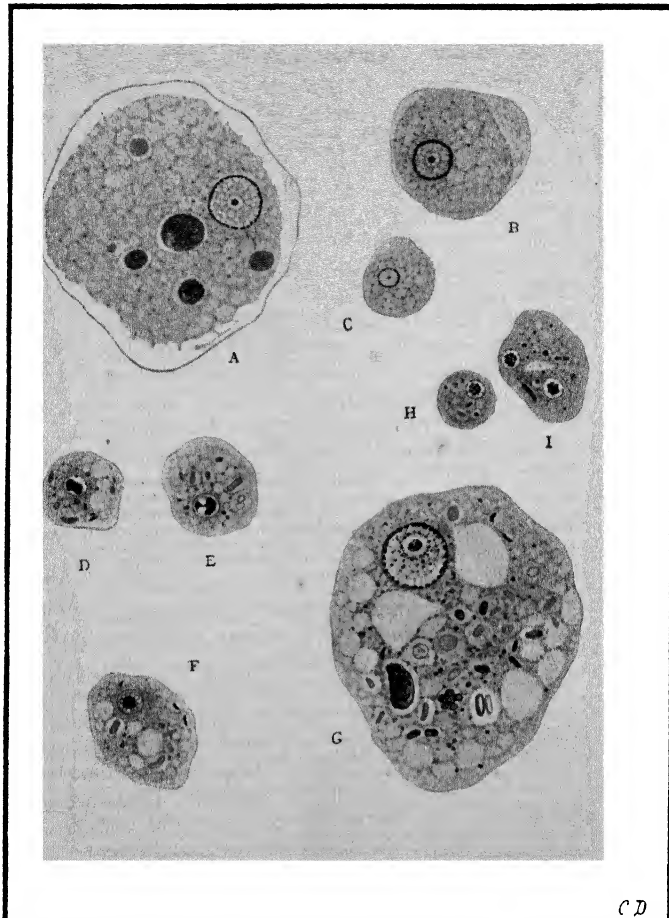


FIG. 183.[1]—Typical specimens of the intestinal amœbæ of man. From fixed and stained preparations. $\times 1500$.

A, B, C. *Entamœba histolytica*. (A) Large tissue-inhabiting form, containing remains of red corpuscles: from stool in acute amœbic dysentery. (B) Precystic form, belonging to a race forming cysts usually 12 μ to 14 μ in diameter. (C) Precystic amœba of a race forming cysts usually 7–8 μ in diameter.
D, E. *Endolimax nana*.
F. *Iodamœba butschlii*.
G. *Entamœba coli*.
H, I. *Dientamœba fragilis*—uninucleate and typical binucleate individuals respectively.

[1] We are indebted to Colonel Byam and Dr. Archibald, and to Mr. Clifford Dobell for permission to use Figs. 183 and 184, which are reproduced from *The Practice of Medicine in the Tropics*.

cells, but accounts given of the process vary. Appearances of division are rarely seen in the stools, and the process occurs mainly, if not exclusively, in the lesions. Dobell, from a study of sections of the lesions in the cat's intestine (*vide infra*), finds that when a cell is going to divide the chromatin increases in amount and becomes disposed in threads and granules, while the nucleus becomes spindle-shaped. It then undergoes constriction and division, each half assuming the circular form, and then division of the cell follows. He was unable to detect distinct chromosomes, and he regards the process as intermediate in character between mitotic and amitotic division.

As the symptoms of the disease abate, the entamœbæ undergo certain changes, which ultimately result in their encystment. The cysts, which are formed when the stools begin to regain their formed character, are spherical and relatively small, measuring 10–15 μ in diameter. (Dobell and Jepps have found that the average size varies in different strains.) The cyst wall is thin, with double contour, and within the cyst four, or sometimes only two, nuclei can be seen in the fresh condition. In fixed and stained specimens the nuclei are seen to have the chromatin at the periphery as in the active amœbæ (Fig. 184, J–P). Beside the nuclei one or more elongated "chromatoid" bodies may be seen, and there are also droplets containing glycogen. Such cysts may be found in the fæces for a long time after dysenteric symptoms have disappeared, and as they are the means of infecting other persons, the individuals passing them are to be regarded as carriers dangerous to the community; such individuals are known as "convalescent carriers." Cysts may be present also in the fæces of those who have never suffered from dysentery—"contact carriers"; in them infection with the entamœba has occurred, and though slight lesions are probably present, they are not sufficient to give rise to symptoms. The occurrence of liver abscess has been recorded in such carriers. In the transition from the active amœboid form to the cystic stage the following changes occur. The amœba becomes smaller and the nucleus more distinct and richer in chromatin, though still maintaining its characteristic features. Further diminution in size occurs, probably by division, the small cell loses its amoboid property, and a hyaline cyst-wall forms around it. The nucleus then divides into two or into four, as the case may be. These transition forms are to be met with in stools which are losing the typically dysenteric character. Unlike the free amœbæ, the cysts have considerable powers of resistance, and in the moist condition may survive

for several weeks outside the body. They represent a resting and resistant stage of the parasite, by means of which fresh infection occurs (*vide* p. 819). It is important to recognise that they are not present in acute cases, and that accordingly infection is spread mainly, if not exclusively, by convalescents and carriers. They are found only in the intestine, and are never present in the secondary abscesses. The cysts remain in the fæces for a long time, and they have been found several years after dysenteric symptoms have disappeared. It is considered by some that they may persist during the rest of the patient's life. In connection with the treatment of amœbic dysentery by emetine, it is to be noted that failure to cure in some cases cannot be ascribed to resistance to the drug on the part of cysts which may be present.

Non-Pathogenic Amœbæ of the Intestine.—As has been mentioned above, four other species of intestinal amœbæ are met with in man. These are *Entamœba coli*, *Endolimax nana*, *Iodamœba*, and *Dientamœba fragilis*. All of them are non-pathogenic commensals. In distinguishing them, the characters of the nuclei and cysts are of most importance. A further type of entamœba has been described by Brumpt and designated *E. dispar*. The following is a short description of these organisms :

The *Entamœba coli*, an intestinal commensal of common occurrence, is of about the same size as *E. histolytica*, but on the whole is a little larger. When at rest it shows no differentiation into ectoplasm and endoplasm ; the nucleus is readily seen, and shows a highly refractile membrane with chromatin lining it and in the interior. The characters of the nucleus, as seen in fixed and stained preparations, are shown in Fig. 183, G. The protoplasm has a granular appearance, and in it there are often small vacuoles containing bacteria, food particles, etc. ; glycogen also is present. During amœboid movement, which is usually sluggish, some delicate processes of ectoplasm come into view, but the characteristic movements described above in the case of the *E. histolytica* are not met with. It is generally stated that red corpuscles are not found in the interior, though bacteria, food particles, etc., are often abundant. Dobell has pointed out, however, that the cultivated amœbæ may ingest red cells as readily as *E. histolytica*. The cellular changes in the encysting of the *E. coli* correspond with those of *E. histolytica*, and the ultimate result is the formation of a fairly large cyst (Fig. 184, V, W), which contains from two to eight nuclei. As seen in the fresh state in fæces, the cysts measure on an average 15–20 μ in diameter, the cyst-wall is distinct and refractile, the protoplasm is granular, and in the interior the nuclei are clearly visible. The nuclei have a similar structure to those of the free amœbæ. Glycogen is abundant in the cysts, but elongated or bar-shaped chromatoid bodies are

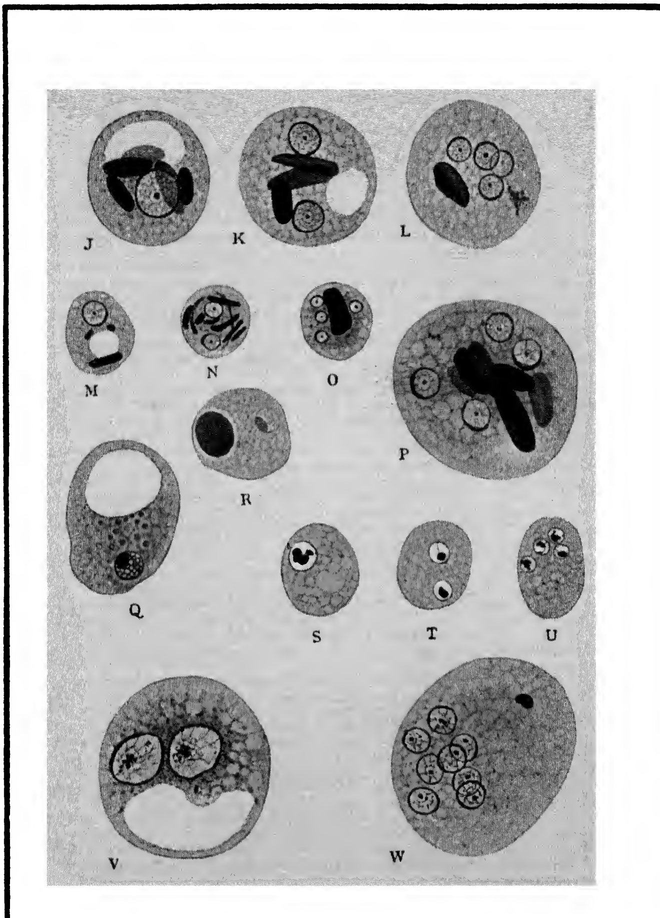


FIG. 184.—Typical specimens of the cysts of the intestinal amœbæ of man. From fixed and stained preparations. ×1500.

J–P. Cysts of *E. histolytica*. J, K, L, uninucleate, binucleate, and quadrinucleate specimens belonging to a strain forming cysts of medium size (mostly 12 μ to 13 μ in diameter); M, N, O, small cysts belonging to a strain with cysts averaging 6 6 μ in diameter; P, large cyst belonging to a strain with cysts of average size 15 μ.

Q, R. Cysts of *I. butschlii*; R, stained with Best's carmine, showing glycogen mass.

S, T, U. *E. nana*—uninucleate, binucleate, and quadrinucleate cysts.

V, W. *E. coli*—binucleate and octonucleate cysts.

(The cyst-walls are not shown, as they are usually invisible in specimens mounted in balsam.)

usually absent. They are in these ways distinguishable from the cysts of *E. histolytica*.

Endolimax nana (formerly called *Entamœba nana*). This is a small amœba about 8–12 μ in diameter and somewhat resembling a small *E. coli* in its appearance and movements. Like the latter, also, it often has in its protoplasm vacuoles containing ingested bacteria. In fixed and stained preparations its small nucleus is characterised by its relatively large karyosome, which varies much in shape and in its position within the nucleus (Fig. 183, D, E). The amœbæ are not infrequently parasitised by a small micro-organism belonging to genus *Sphærita*, the small oval and highly refractive spores of which form regular cluster-like masses of cocci (Dobell). The cysts of *E. nana* are of about the same size as the free forms, and when mature contain four minute nuclei, the karyosome in each having the peculiar character referred to (Fig. 184, S, T, U). They may, in addition, contain glycogen and small granules or rods. *E. nana* is a common commensal of the bowel, and has a wide geographical distribution ; it has often been found in this country in those who have never been abroad. Though it has been met with most frequently in conditions of intestinal disorder, it also occurs in quite healthy individuals, and there is no evidence that it has any pathogenic properties. Thomson and Robertson have recently obtained cultures of *E. nana* in the medium of Bœck and Drbohlav (*vide infra*), in which it grows fairly well and presents the same features as in the fæces. No cysts were observed in the cultures.

Iodamœba bütschlii.—As seen in the fresh fæces, the organisms measure 8–12 μ in diameter and resemble somewhat small specimens of the *E coli.* As in the latter, there is little differentiation between ectoplasm and endoplasm, and within the endoplasm there are many vacuoles containing ingested bacteria, etc. The nucleus is vesicular and contains a large central karyosome (Fig 183, F). The cysts are about the same size as the free forms. Each cyst contains a single nucleus in which a similar karyosome is situated at the periphery, and usually also a comparatively large mass of glycogen (Fig. 184, Q, R). This species is of less common occurrence than the two previously described.

Dientamœba fragilis (Jepps and Dobell).—This organism, which is rare, is distinguished by usually possessing two nuclei of similar structure, though uninucleated forms also are met with. It is of small size, measuring 5–11 μ in diameter. Each nucleus (1·5–2 μ in diameter) is vesicular and contains a central karyosome of granular structure with a clear zone around it (Fig. 183, H, I). The cysts of this organism have not yet been found.

Entamœba dispar.—Brumpt has given this name to an entamœba which cannot be differentiated morphologically from the smaller forms of *E. histolytica* and which also produces quadrinucleate cysts. It is stated to be non-pathogenic for man and also to be incapable of producing in kittens the severe lesions which are caused by *E. histolytica.* It is impossible at present to state whether it is a separate species or merely a permanently attenuated form of *E. histolytica.*

Cultivation.—Cultures of amœbæ in association with various bacteria were obtained by several workers, *e.g.* Lesage, Mus-

grave and Clegg, Noc and others, from cases of dysentery and also from various sources outside the body, but in no instance could the amœba grown be established as identical with *E. histolytica*; and the view was gradually gaining ground that the latter was a strict parasite, not cultivable outside the body. In 1925, however, Boeck and Drbohlav published an account of successful cultivation on a medium composed of Locke's solution, egg, and serum or albumin (*vide infra*). Growth was obtained by inoculating the medium with material from the stools of two cases of dysentery, and subcultures, made by transferring some fluid from the deep part of the culture tube by means of a glass pipette, were maintained through many generations—in the case of one strain for over eight months. The amœbæ in cultures were found to be comparatively short-lived, dying out after four or five days, and subcultures were made every two days. The formation of cysts in cultures was noted on only one occasion. On injecting kittens *per rectum* with the subcultures, Boeck and Drbohlav were able to produce typical dysenteric lesions, this result being obtained in eleven out of sixteen kittens used. In two of these, liver abscesses also developed. The inoculation experiments were carried on over a period of five months of subculturing, and there was no evidence that the amœbæ became diminished in virulence (Dobell and Laidlaw, however, found that on subculturing loss of virulence might finally occur). These cultural results have been confirmed by Thomson and Robertson, and by Dobell and Laidlaw, who carried out a long series of subcultures, and observed also the formation of cysts from time to time. The latter observers found that the addition of sterile solid rice-starch to the medium of Boeck and Drbohlav gave more luxurious and prolonged growth of the amœba, and also enabled the whole life cycle, including excystation from the cysts, to be observed *in vitro*.

Medium of Boeck and Drbohlav.—Four eggs are washed, brushed with alcohol, and broken into a sterile flask containing glass beads; 50 c.c. of Locke's physiological solution are then added, and the mixture is broken up by shaking. Sufficient of the mixture is added to test-tubes so that slants 1–1½ inches in length may result upon coagulation by heat. The tubes are slanted in an inspissator and heated at 70° C. until the mixture has solidified. They are then transferred to the autoclave and sterilised. The medium in each tube is covered to a depth of 1 c.c. above the slant with a mixture composed of eight parts of sterile Locke's solution and one part of sterile inactivated human blood serum. The tubes are then incubated to determine sterility. In place of the human serum a solution of crystallised egg albumin may be substituted. A

1 per cent. solution of the albumin in Locke's solution is prepared and sterilised by passing through a Berkefeld filter. It is then added to the tubes containing the egg slants as above described. The initial reaction of these media varies from pH 7·2 to 7·8 and requires no adjustment.

Dobell and Laidlaw's modification.—Slopes of inspissated horse serum are prepared in tubes and covered with egg albumin solution as described above. A small amount of sterilised rice starch is then added.

Distribution of E. histolytica.—As already stated, the organisms are usually found in large numbers in the contents of the large intestine in amœbic dysentery. Their real habitat, however, is the tissues, where they exert a well-marked action. The lesions are chiefly in the large intestine, especially in the rectum and at the flexures, though they may also be present in the lower part of the ileum. At first there are local swellings on the mucous surface, chiefly due to inflammatory gelatinous œdema with little leucocytic infiltration; soon, however, the mucous membrane becomes partially ulcerated, more or less extensive necrosis of the subjacent tissues occurs, and gangrenous sloughs result. The ulcers thus come to have irregular and overhanging margins, and the excavation below is often of wider extent than the aperture in the mucous membrane. The amœbæ are found in the mucous membrane when ulcers are being formed, but their most characteristic site is beyond the ulcerated area, where they may be seen penetrating deeply into the submucous and even into the muscular coats (Fig. 185). In these positions they may be unattended by any other organisms, and the tissues around them show œdematous swelling and more or less necrotic change, without much accompanying cellular reaction beyond a certain amount of swelling and proliferation of the connective-tissue cells. The amœbæ appear to produce a digestive softening of the tissues, hence the term "histolytica" is an appropriate one. These lesions are characteristic of amœbic dysentery.

As a complication of this form of dysentery, liver abscess is of comparatively common occurrence. It is usually single and of large size, but more than one may be present. The contents are usually a thick chocolate-coloured fluid of somewhat slimy consistence, which is largely composed of necrosed and liquefied tissue with admixture of blood in varying amount. In the wall of the abscess the amœbæ can be found, and usually are the only organisms present; occasionally secondary invasion by pyogenic organisms occurs. The amœbæ are most numerous at the spreading margin, and this probably explains a fact

pointed out by Manson, that examination of the contents first removed may give a negative result, while they may be detected in the discharge a day or two later. The action here on the tissues is of an analogous nature, namely, a necrosis with softening and partial liquefaction, attended by little or no suppurative change. There is, however, evidence that the amœbæ may infect the liver without causing actual abscess formation, merely a hepatitis, and that this may be followed by cirrhosis. Abscesses are also met with in the lungs, as when a liver abscess has ruptured into the lung, which not very infrequently happens, but in such cases the amœbæ have not been found in the sputum. There have also been recorded a considerable number of cases of cerebral abscess in which the amœbæ have been found; most of these have been secondary to lung infection.

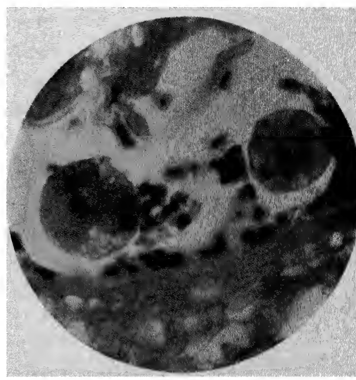


FIG. 185.—Section of large intestine from a case of acute amœbic dysentery. Two specimens of *E. histolytica* are seen within a blood vessel in the muscular coat beneath the floor of an ulcer. Stained with iron hæmatoxylin. ×600.

Experimental Inoculation.—Dysentery develops occasionally in animals, *e.g.* in monkeys, but it is of comparatively rare occurrence. The disease may sometimes be reproduced in the dog by experimental inoculation with dysenteric material, as was shown first by Lösch and afterwards by Kartulis, the latter producing the disease in one instance with the contents of a tropical abscess. Cats are, however, found to be more susceptible, especially young animals, and have been mainly used in investigation. Dysentery follows readily the introduction into the rectum of mucus from a human case, especially when the bowel is plugged; and a similar result has been obtained by means of material from a liver abscess containing amœbæ (Kruse and Pasquale). Infection may take place also by oral administration, but in this case the presence of cysts in the material is essential since the amœbæ are destroyed in the stomach, as was first shown by Quincke and Roos. The experimentally produced disease in cats is of an acute character and

is usually fatal; in conformity with this, the cystic stage of the organism is not met with in the stools (*vide supra*). The most important experiments, however, were those carried out by Walker and Sellards on the human subject. They administered to Filipinos, who acted as volunteers, various amœbæ and entamœbæ or their cysts, the material being mixed with magnesium oxide or starch, and enclosed in gelatin capsules. Of twenty volunteers fed with motile *E. histolytica* or cysts, seventeen became parasitised after one feeding, the amœbæ appearing in the fæces after one to forty-four days (the average being nine days); of the remaining three one was fed repeatedly and became infected after the third occasion. The amœbæ or cysts persisted for an indefinite period in the stools of those who became infected; four of them contracted dysentery, the average period of incubation being sixty-five days. In the case of the other amœbæ cultivable at that time, they found that though the organisms might be detected in the fæces after feeding with them, none of them became parasites and no pathogenic effects were produced. These results are of great importance both in demonstrating the specific pathogenic properties of the *E. histolytica*, and also in showing that it may become an intestinal parasite without causing dysenteric symptoms and lesions. Walker and Sellards concluded that the *E. histolytica* is a strict parasite, and that the source of infection is always another individual harbouring the organism in the intestine, and this view has received general support. An extensive epidemic of amœbic dysentery occurred in the United States in 1933 which was traced to the water supply of two hotels in Chicago being infected from communication with the sewers. The original source of infection was not discovered, however. The incubation period in this epidemic was usually about two weeks, but in some cases dysentery developed within a week and in others not till after three or four months. Wenyon and O'Connor examined the stools of nearly two thousand healthy British soldiers in Egypt and found that there was infection by *E. histolytica* in fully 5 per cent., while in less than a sixth of the infected individuals was there a history that they had suffered from dysentery. Among Egyptian natives 13·5 per cent. were found to be carriers of *E. histolytica* cysts. Observations made in more recent times show that *E. histolytica* has a much wider distribution than was formerly supposed. Yorke, Malins Smith, and others, for example, have found that the cysts are not infrequently to be found in various classes of the population in this country, the largest percentage of positives

being among asylum inmates. The extensive investigations of Dobell have shown that several species of *Macacus* monkeys in an apparently healthy state harbour naturally in their intestine amœbæ which in all their microscopic and cultural characters are identical with *E. histolytica*. Kittens were infected either by feeding with cysts contained in the monkeys' fæces or by intrarectal inoculation with cultures from the latter; they acquired a subacute or chronic form of the disease, which points to the virulence of the monkeys' amœbæ being relatively low. Also *Macacus* monkeys which had been freed from their natural infection by emetine were successfully re-infected by feeding with cyst-containing cultures of *E. histolytica* derived from other species; these cultures had been growing *in vitro* for up to one and a half years. Amœbæ appeared in the fæces within four days. The infections thus produced were permanent, but no lesions developed. Similarly a culture derived from a man with acute amœbic dysentery proved infective for monkeys five years after isolation. According to the views of Brumpt the high proportion of European populations showing these cysts in the absence of cases of amœbic dysentery is due to the fact that they are infected with the non-pathogenic *E. dispar* (*vide supra*).

In the case of the *E. coli*, Walker and Sellards were able to bring about parasitism by feeding with the cysts of the organism, but no pathogenic effects followed. Their results accordingly confirm the view previously held that it is a harmless organism. It has practically a world-wide distribution, and in certain countries is very common. Schaudinn found that in East Prussia as many as 50 per cent. of the population were infected with it, and confirmatory results with regard to its common occurrence were obtained by Craig in San Francisco.

Methods of Examination.—The fæces in a suspected case of acute dysentery ought to be examined microscopically *as soon as possible after being passed*, as the amœbæ disappear rapidly, especially when the reaction becomes acid. A drop of blood-stained mucus is placed on a slide without the addition of any reagent, a cover-glass is placed over it but not pressed down, and the preparation is examined in the ordinary way or on a warm stage, preferably by the latter method, as the movements of the entamœbæ become more active. The addition of a solution of neutral red, 1 : 5000, is recommended by some, as it stains the amœbæ a pale pink colour. In the examination for cysts, when the fæces have more of a formed character, a small portion of fæces is emulsified in saline or in Lugol's iodine solution, which brings out the nuclei rather more distinctly and stains glycogen granules. The cysts may be conveniently picked out by means of a dry lens and then

examined under the oil immersion. In this case immediate examination of the fæces after being passed is not essential, as the cysts persist unchanged for several days.

For permanent preparations dried films are not suitable, as in the preparation of these the amœbæ become distorted. Wet films should be used, and corrosive-alcohol is a very suitable fixing agent (p. 111). For such films Heidenhain's iron hæmatoxylin has been found to be one of the best stains, but ordinary hæmalum gives quite good results.

In sections of tissue the entamœbæ may be stained by methylene-blue, by safranin, by hæmatoxylin and eosin, iron hæmatoxylin, etc. Benda's method of staining with safranin and light-green is also a very suitable one. Sections are stained for about an hour in a saturated solution of safranin in aniline water (the latter is prepared by adding 5 c.c. aniline to 100 c.c. distilled water in a flask, shaking vigorously, and filtering through a filter paper previously moistened with water; the solution should be kept in the dark); they are then washed in water and decolorised in a ½ per cent. solution of light-green in alcohol till most of the safranin is discharged, the nuclei, however, remaining deeply stained. In this method the nuclei of the entamœbæ are coloured red (like those of the tissue cells), the protoplasm being of a purplish tint.

Methods of cultivation have been described above.

Entamœba gingivalis

The occurrence of amœbæ in the mouth has been recognised for a considerable time, but special attention has been directed to them more recently, in view of their occurrence in pyorrhœa. The amœbæ, so far as is known, all belong to the one species—*E. gingivalis* (also called *E. buccalis*). The organism is a small amœba which tends to abound in certain morbid conditions of the mouth. It is of smaller size than the *E. coli*, measuring about 10–20 μ in diameter, while its nucleus, seen with some difficulty in the living amœba like that of *E. histolytica*, is relatively small, measuring 3–4 μ. The amœbæ show active amœboid movements, throwing out rounded pseudopodia, and ectoplasm and endoplasm are clearly distinguishable. The endoplasm is granular, and often contains numerous rounded bodies or inclusions, which stain deeply with nuclear dyes, and have been supposed by some to represent the remains of nuclei of leucocytes or other cells. No cysts of this organism have been observed. Although the amœbæ have been observed in a fair proportion of healthy individuals, there is no doubt that they are specially abundant in morbid states. They are usually present in pyorrhœa, and are commonly met with in dental caries, and in the tartar of the teeth. It has not been established that the organism is responsible for pyorrhœa or any other morbid change, and the evidence goes to show that it is a fairly common commensal of the mouth, which becomes more numerous in pathological conditions.

CHAPTER XXVII

PATHOGENIC PROTOZOA (*continued*): TRYPANOSOMES, LEISHMANIAE, ETC.

THE PATHOGENIC TRYPANOSOMES

THE trypanosomes are protozoal organisms belonging to the sub-class Flagellata; many members of the genus live in the blood and tissues in various animals and cause important disease conditions. These diseases have general resemblances to one another. They tend to be characterised by wasting, cachexia, anæmia, fever, often of an intermittent type, and irregular œdemas, and frequently have a fatal result—the condition being called nagana. In many cases the infective agent in trypanosomiases of mammals has been proved to be conveyed from a diseased to a healthy animal by the agency of blood-sucking insects (especially tsetse flies). Under experimental conditions infection can often be transmitted by injecting into a normal animal blood containing the parasites. In the human subject three species of trypanosomes are met with, *T. gambiense*, *T. rhodesiense*, and *T. cruzi*.

Morphology and Biology of the Trypanosomes.—If a drop of fresh blood containing trypanosomes be examined between a slide and coverslip, the organisms are seen to be fusiform, one end passing into a pointed flagellum. In the living condition the trypanosomes are usually actively motile by an undulatory movement of their protoplasm and a lashing of the flagellum, but many species show little tendency to movements of progression. From the fact that in progression the flagellum is in front, the end at which it emerges is regarded as anterior. The size varies, but many, including those parasitic in man, are about 12–35 μ long and about 1·5 to 3 μ broad. The method of examining the fresh blood is the one most likely to reveal the presence of trypanosomes, if these are present in small numbers, since they are readily detected by the movement which they impart to the red corpuscles round about. But the minute structure of the organisms can be studied in preparations fixed

after drying or preferably when wet, and stained by Romanowsky dyes, such as those of Leishman or Giemsa.

For staining trypanosomes (or Leishman-Donovan bodies) *in sections* so as to bring out the chromatin structures, Leishman recommended the following method after the section has been brought into distilled water. The excess of the latter is blotted off ; a drop of fresh blood serum is then placed on the preparation and allowed to soak in for five minutes. The excess is removed by blotting, and the remainder is allowed to dry on the section, which is now treated with a mixture of two parts of Leishman's stain and three of distilled water, and placed in a Petri dish for one to one and a half hours. Decolorisation and differentiation are effected by alternately applying dilute acetic acid and caustic soda solutions (*vide* p. 130).

In preparations stained by the above methods the protoplasm of trypanosomes stains blue, and in certain species some parts are more intensely coloured than others. Sometimes it contains violet-coloured granules (chromatin granules), and occasionally there appears in it slight longitudinal striation. Two bodies are always present in the protoplasm. An oval granular body staining purple, the trophonucleus or macronucleus, is usually near the middle, and towards the posterior end is a minute intensely stained purple granule known as the kinetonucleus, kinetoplast, or micronucleus, which may be differentiated into the blepharoplast (the granule from which the axoneme arises —*vide infra*), and deeply staining material, the parabasal body (the micronucleus was held by Laveran to represent the centrosome from the analogy of appearances in certain spermatozoa which closely resemble trypanosomes in structure). The micronucleus is often surrounded by an unstained halo, and in its neighbourhood, in certain species, a vacuole has been described. From the micronucleus or from its neighbourhood there arises an important structure in the trypanosome—the undulating membrane. This is of varying breadth, has a sharp, undulating, free margin, and surmounts the protoplasm of the organism like a cock's comb ; it narrows towards the anterior end, where it becomes the flagellum. A filament (axoneme) which stains of the red tint of chromatin, commences at the blepharoplast and runs along the free margin of the undulating membrane ; it then forms the core of the flagellum. The latter is continuous with the protoplasm of the body of the organism. Motion is chiefly effected by the undulations of the membrane and of the flagellum. In different species of trypanosomes variations occur in shape, in length, in breadth, in the position of the micronucleus (and therefore in the length of the undulating membrane), in the

breadth of the membrane, in the length of the free part of the flagellum, in the shape of the posterior end, which is sometimes blunt, sometimes sharp, and in the presence or absence of free chromatin granules in the protoplasm.

Multiplication in the body fluids ordinarily occurs by longitudinal, amitotic division, the parasite having increased in length and breadth beforehand. First of all, the micronucleus divides, sometimes transversely, sometimes longitudinally, then the macronucleus and undulating membrane, and lastly the protoplasm. In some species only the root of the flagellum divides, so that in the young trypanosomes the flagellum is short and subsequently increases in length (*T. lewisi*). It has been held that the whole flagellum takes part in the general splitting of the organism ; but, according to Wenyon, after the blepharoplast has divided, the original axoneme remains attached to one moiety and a new axoneme grows out of the other. The view has been advanced that in certain cases reproduction occurs by the formation of "latent bodies" consisting of the nucleus fused with the blepharoplast (Moore and Breinl), or by the endogenous formation in the nucleus of "chromidial buds" (Minchin), or "infective granules" (Henry and other observers), which when extruded from the protoplasm develop into trypanosomes ; but according to other workers these appearances may be due to degenerative changes.

The morphology of certain species of trypanosomes tends to vary, and this feature is more or less marked according to the host which they are infecting. In man both *T. gambiense* and *T. rhodesiense* are practically monomorphic, but when transferred to laboratory animals they become polymorphic and in the circulating blood show differences in shape and size. There is a form long and slender in both body and nucleus, the free part of the flagellum being longer than the body and the protoplasm devoid of granules. At the same time a broader form of the organism with a larger and rounder nucleus and a blunter posterior extremity is found; its undulating membrane is narrow and the free part of the flagellum is shorter than the body, and the protoplasm contains granules. According to one view, this polymorphism indicates sexual differentiation, the former being the male form and the latter the female, but intermediate forms are also met with and conjugation has never been observed. Accordingly, it is probable that the variations indicate merely different stages of growth. Whether any pathogenic significance is to be attached to the occurrence of these different forms is at present unknown. In the case of

T. rhodesiense especially, posterior nuclear forms are met with in which the trophonucleus is situated toward the posterior end of the parasite close to the blepharoplast.

Infectivity and Pathogenicity for different vertebrates.—Some trypanosomes, such as *T. lewisi* and *T. vivax*, will infect only a single vertebrate species or a very limited number either under natural conditions or experimentally. Others can produce infections in most species of domestic and laboratory mammals, e.g. *T. brucei*, *T. gambiense* and *T. rhodesiense*, both through the agency of their insect vectors and also by injection of blood containing the parasites. While some species of trypanosomes give rise to serious disease, in the case of others a heavy infection may occur without the animal suffering any apparent inconvenience; again, a form producing disease in one species of host, may be present in considerable numbers in another species without causing any pathogenic effects. Certain species, e.g. *T. brucei*, when passed repeatedly through rats or mice, by injecting parasite-containing blood, become highly virulent for the specific host and increase progressively in the blood, thus producing a rapidly fatal infection with a septicæmic course. On the other hand, in rabbits a chronic relapsing infection is produced by several of the pathogenic trypanosomes, which is similar to the clinical disease in the larger domestic animals and man. The capacity to acquire virulence for a specific host is a property inherent in the trypanosome; thus *T. rhodesiense* is more virulent for laboratory animals than *T. gambiense*, but inferior in this respect to *T. brucei* when fully accommodated and virulent.

In chronic infections the number of organisms present in the peripheral blood varies, and thus the potentiality of infection by means of an invertebrate carrier also varies. When the organisms are absent from the blood they may still be found in the solid organs and in the bone marrow, and in such situations may go through a phase of development. In *T. cruzi* such a stage has been demonstrated in tissue cells.

The means by which trypanosomes produce their chronic pathogenic effects are unknown. Small animals with a septicæmic form of the infection tend to die suddenly with convulsions. The blood sugar in such animals is low, and as the parasites have an active sugar metabolism, it is possible that this accounts for the hypoglycæmia and contributes to the fatal result. Toxic substances are stated also to have been obtained from the bodies of the parasites.

Transmission.—The outstanding fact in the biology of the

pathogenic trypanosomes is that infection from vertebrate to vertebrate usually takes place through the agency of biting or blood-sucking insects, or, in the case of frogs and fishes, by leeches. The mere mechanical transference by such invertebrates is possible, and in certain cases multiplication of the organisms in the biting apparatus of the invertebrate occurs. Such a mechanical or semi-mechanical transference, however, plays only a subsidiary part in spreading the infection in many cases, including the human trypanosomes, since a considerable period elapses before an insect which has ingested infected blood becomes infective for new hosts. Here the parasite undoubtedly goes through a cycle of development within the invertebrate, the details of which are in some instances as yet undetermined. In the alimentary tract of the insect, the trypanosomes are seen to undergo modifications in form. They may show simple division, by which the resulting individuals become smaller; the relation of kinetonucleus and trophonucleus may be altered, the former coming to lie anterior to the latter, while the undulating membrane and flagellum become rudimentary (crithidial forms). In other cases, organisms resembling *Leishmaniæ* result. Koch and Kleine also found in the intestine agglomerations of immature forms which they ascribed to the results of sexual conjugation. The behaviour of the organisms in the invertebrate host before they again become infective for vertebrates varies in different instances. Thus *T. gambiense* passes finally to the salivary glands of the tsetse fly; on the other hand, *T. lewisi* travels to the rectum of the rat flea and the infective forms pass out in the fæces. While the analogy of what happens in the malarial parasite suggests the possibility of a sexual element in a trypanosomal cycle, there is at present no definite proof that such a stage has ever been observed. The trypanosomes are not transmitted hereditarily by infected insects. Both sexes act as the vectors.

Cultivation.—A number of the trypanosomes have been cultivated outside the bodies of their natural hosts, the first work having been done with the rat trypanosome, *T. lewisi* by Novy and MacNeal, who introduced a special medium for the purpose.

Nicolle's modification of Novy and MacNeal's blood agar ("N.N.N. medium") for the cultivation of trypanosomes, leishmaniæ, etc., is prepared as follows: Mix thoroughly in a flask 14 grams agar, 6 grams NaCl, and 900 c.c. water, and steam for two hours (the medium is not neutralised or cleared). Filter through cotton wool

and tube 2 c.c. in tubes of $\frac{5}{8}$-inch diameter; autoclave at 120° C. for twenty minutes. Cool the medium to 50° C. in a water bath; then into each tube introduce 20 drops of whole rabbit's blood obtained with aseptic precautions as described on p. 68. Mix thoroughly by "rolling" in the hand, and allow the medium to solidify in the sloped position. Incubate the tubes for several days at 37° C. to test sterility. As the presence of abundance of condensation water is essential, evaporation must be prevented by capping the tubes. Inoculation is made by introducing the infective material by means of a capillary pipette into the water of condensation.

According to Behrens a more suitable medium for the growth of *T. brucei* is prepared as follows: 125 grams chopped beef are digested with 250 c.c. water overnight; the extract is strained, boiled, and filtered. The filtrate is then dialysed in a collodion sac for twenty-four to forty-eight hours against running distilled water and finally made up to 1000 c c. There are then added 2 per cent. peptone, 0·5 per cent. NaCl, 0·01 per cent. $CaCl_2$, 1 per cent. N/1 Na_2CO_3, and 2 per cent. agar; solution is effected by heating. The medium is tubed in amounts of 1 c.c. and autoclaved at 105° to 108° C. for fifteen minutes. Shortly before use it is melted and cooled to 60° C. and two volumes of defibrinated rabbit's blood are added; the whole is well mixed and sloped.

In cultures, the organisms may divide longitudinally, as seen in the blood, or crithidial or leishmania forms may result, the former being often arranged in rosettes containing a large number of individuals with their flagella pointing in one direction. A fresh infection mav sometimes be originated by introducing such cultures into suitable animals. But in the case of the human trypanosomes cultures are difficult to establish and usually are not infective.

Identification of trypanosome species.—The differentiation of species of trypanosomes may present great difficulties. Both morphological and biological characters must be taken into account. As has been seen, *T. gambiense* and *T. rhodesiense* are similar in their appearance in human blood, but differ in experimental animals, the latter alone showing frequent posterior nuclear forms. Again, *T. gambiense* differs from all other morphologically similar trypanosomes in being insusceptible to the trypanocidal action of human serum. Biological characters which are important in this respect, are the species of susceptible vertebrates and the course of the disease in these; the species of the insect vectors; the behaviour of the parasites both in vertebrate and invertebrate hosts; and the response of infected animals to administration of trypanocidal drugs. Immunity tests may not be of value, since trypanosomes are capable of great variation, and parasites derived from a relapse are frequently serologically different

from those of the strain causing the original infection (cf. relapsing fever spirochætes, p. 687).

Human Trypanosomiasis

Sleeping Sickness.—Since the year 1800 the disease called sleeping sickness, sleeping dropsy, or negro lethargy has been recognised as prevailing on the West Coast of Africa from the Senegal to Lagos, and in the parts lying behind the coast between these regions. It has also been found to be rife from Cameroon to Angola and in the Congo valley, and to a less extent up the Niger and its tributaries. In 1901 it began to appear in the Uganda Protectorate, where it has wrought very serious havoc among the native population, and the investigations carried on in that region have led to a knowledge of its cause. After an incubation period of about a fortnight following the bite of an infective fly fever develops; the evening temperature may be elevated several degrees. There may also be headache, hyperæsthesia, and indefinite pains about the body, the pulse tends to be soft and rapid, and in a very large number of cases the superficial lymphatic glands are enlarged. The disease is characterised in the early stages by a change in disposition leading to moroseness, apathy, disinclination for work or exertion, and slowness of speech and gait. In a rapid case lethargy soon develops and deepens; fine tremors, especially of the tongue and arms, appear; progressive emaciation occurs; blood changes arise, consisting of a progressive diminution of the red cells and of the hæmoglobin, and of a lymphocytosis in which the percentage of both the large and small mononuclear cells is increased, so that the former may constitute from 20 to 30 and the latter from 30 to 40 per cent. of all the white cells present. As the disease progresses the drowsiness increases till the individual lapses into a coma from which he cannot be roused. Often during the disease there occur irregular œdematous patches on the skin, and sometimes erythematous eruptions, and effusions into the serous cavities. The disease is an extremely fatal condition, and probably no case where the actual lethargy is developed ever recovers. Not every case runs a progressively advancing course such as that described above. Sometimes the chief early feature, besides enlargement of glands, is the occurrence from time to time of attacks of fever which may be mistaken for malaria, and from these apparently complete recovery may take place; recurrence, however, follows as a rule, and ultimately the typical terminal

phenomena may commence. Such cases may go on for years, and it is probable that many patients die of pneumonia without exhibiting typical manifestations of the malady from which they really suffer.

As described by Mott, the most striking anatomical change is the presence of a chronic meningo-encephalitis and meningo-myelitis. The pia-arachnoid is sometimes opaque and slightly thickened and may be adherent to the brain, and its vessels usually show some congestion. The sub-arachnoid fluid is sometimes in excess and occasionally may even be purulent. The membranes of the spinal cord show similar changes. The chief other feature is the presence of enlarged lymphatic glands in the body, but otherwise there is nothing special to note. With regard to the microscopic changes, the chief feature is a proliferation and overgrowth of the neuroglia cells, especially of those which are related to the sub-arachnoid space and also the perivascular spaces, with accumulation and probably proliferation of lymphocytes in the meshwork. The changes in the lymph glands are of similar nature, and resemble the infiltration of the perivascular spaces of the central nervous system. These changes are specially significant in view of the lymphocytosis present in the blood, which has already been noted, and which so often occurs in protozoal infections. In the nerve elements there are merely some atrophy of the dendrons of the nerve cells, a diminution of Nissl's granules, and an excentric position of the nuclei.

Trypanosoma gambiense.—The first case in which trypanosomes were found in the blood was recorded by Dutton in 1901; the patient, who was not obviously suffering from sleeping sickness, was a European then living at Bathurst on the Gambia. The progress of the disease was very slow, and was characterised by general wasting and weakness, irregular rises of temperature, local œdemas, congested areas of the skin, enlargement of spleen, and increased frequency of pulse and respiration; death occurred a year after the case came under observation, after an access of fever, and a striking fact was the absence of any gross lesion. During the time the patient was under observation trypanosomes were repeatedly demonstrated in the peripheral blood, and they also developed in monkeys and white rats inoculated with the blood. Pursuing further inquiries, Dutton and Todd demonstrated similar parasites in other Europeans and in several natives in the Gambia region, while about the same time Manson reported a case of the same kind from the Congo. It thus came to be recognised that in man there occurred a disease

somewhat resembling nagana, in which trypanosomes could be demonstrated in the blood, and this was usually referred to as human trypanosomiasis, or trypanosome fever—the trypanosome being named the *T. gambiense.* Since *T. gambiense* usually occurs only in small numbers in the blood in man, its microscopic characters have been studied mainly in infections of animals such as guinea-pigs or rats. In these it measures on the average 15–30 μ, the nucleus is central and the blepharoplast close to the posterior end. The trypanosome is polymorphic, long slender forms with flagella and short broad forms without free flagella, as well as forms of intermediate length, being found (Fig. 187, 1–5). In man the parasites are as a rule scanty in the blood (Fig. 186). They penetrate into the tissues, especially muscle.

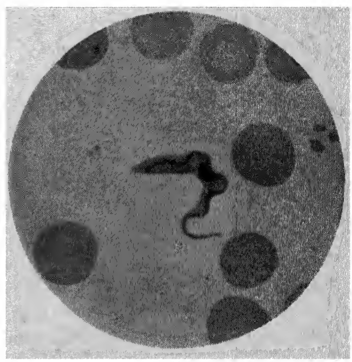


Fig. 186. — *Trypanosoma gambiense* from blood of human case. Leishman's stain. ×1000. See also Plate VI., Fig. 26.

Owing to the seriousness of the epidemic of sleeping sickness in Uganda, a Commission of the Royal Society was dispatched in 1902 to investigate the condition. Castellani found in some cases in the cerebro-spinal fluid, especially when this was centrifuged, living trypanosomes resembling the *T. gambiense*, which seem at first to have been regarded as accidental. Bruce, pursuing the work of the Commission with Nabarro and Greig in 1903, made a series of examinations in several infected localities, and demonstrated the trypanosome in every case of the disease. This work formed the starting-point for inquiries, which proved that the parasite is the causal agent of the condition. The organisms were not seen in the cerebro-spinal fluid of patients dying of other diseases in the sleeping sickness area. On the other hand, it was found that if cerebro-spinal fluid withdrawn from cases of the disease was injected into monkeys (especially *Macacus rhesus*), trypanosomes appeared in the blood, and in many cases in three or four months the animals died of an illness indistinguishable from sleeping sickness, and with the parasites in the central nervous system. On inoculation of other species of animals, *e.g.* herbivora or the

guinea-pig, in nearly every case a proliferation of the parasite takes place, as indicated by its appearing in the blood; but often either no disease occurs or this runs a very chronic course. Small animals such as rats and mice can also be infected, *e.g.* by subcutaneous inoculation. Under ordinary conditions the trypanosomes do not long survive removal from the body.

It was found that in the parts round the north end of Victoria Nyanza where sleeping sickness was prevalent, the distribution of the disease exactly corresponded with the distribution of a blood-sucking insect, the *Glossina palpalis*, a species closely allied to the *Glossina morsitans* of nagana. When one of these flies was fed on a sleeping sickness patient and then allowed to bite a monkey, trypanosomes frequently appeared in the animal's blood, and the same result often followed when fresh flies caught in the sleeping sickness area were placed on a monkey. Kleine established the important fact that when *Gl. morsitans* was allowed to bite an animal suffering from nagana it did not become infective for about twenty days. This was confirmed for *Gl. palpalis*, in the case of monkeys infected with *T. gambiense*, by Bruce and those associated with him. Here it was found that infectivity did not appear till about thirty-two days after the fly had fed, and continued until at least seventy-five days. It was at first supposed that monkeys could not be infected with the trypanosomes from the bruised-up bodies of the fly, but Bruce succeeded in originating an infection with this material, results being positive during the first two days after the fly had bitten and then being negative till after the twenty-second day. Bruce noted that the renewed infectivity corresponded with the appearance of "metacyclic" trypanosomes in the salivary gland of the glossina. The cycle of development of the trypanosome in *Gl. palpalis* has been found by Bruce and his co-workers and by Robertson to be as follows. Thirty-six to forty-eight hours after ingestion of the infected blood many of the parasites are degenerated, but some of the broad forms are dividing; the products of division show crithidial[1] forms only for a short time. At the tenth day numerous trypanosomes of very variable shape and size are present; later, slender forms appear in increasing numbers, and these pass to the proventriculus and hypopharynx. They travel along the duct to the salivary gland, and here crithidial forms develop; the latter again produce trypanosomes, and after the appearance of these "metacyclic" forms at about

[1] In the crithidia the blepharoplast is situated anteriorly and the undulating membrane is only slightly developed.

the twentieth day the fly becomes infective for the vertebrate host. Only a small proportion of flies which have ingested trypanosomes in the blood of a patient become infective subsequently. The temperature has an important influence on the development of the trypanosomes in the fly ; thus Kinghorn and Yorke found in the case of *T. rhodesiense* that under 24° C. the trypanosomes did not invade the salivary glands of *Glossina morsitans*, and hence the fly did not become infective. Certain facts having a serious bearing on the continued infectivity of a locality have emerged. It was found that a certain island on Lake Victoria Nyanza, which had been cleared of infective natives two years previously, still harboured infective flies. To account for this it must be supposed either that the *Glossina* has an extended duration of life, or that the trypanosome exists among the wild animals. It has been found that cattle and wild herbivora, especially certain antelopes, can be infected with the parasite, and can through the medium of the fly infect monkeys. It is possible that such animals, while not suffering in any serious way themselves, are the means of maintaining infectivity. There is no definite evidence that, as Koch supposed, the crocodile harbours the trypanosome.

There is evidence that in certain districts the infection is transmitted by *Glossina tachinoides* and possibly by *Glossina morsitans*.

Early in the Uganda investigations the question arose as to whether the trypanosome of sleeping sickness was different from that discovered by Todd. This was forced on the inquirers by the fact that a large proportion of the natives in the sleeping sickness area were found to harbour trypanosomes in their blood, although not apparently suffering from the disease. Several cases were carefully examined in which trypanosomes were constantly present in the blood, but in which the patients from time to time suffered from fever, and during these pyrexial periods trypanosomes were found in the cerebro-spinal fluid. It was suggested that these cases were on the way to develop sleeping sickness. A very important observation was that while in sleeping sickness areas a large proportion of the native population harboured trypanosomes, this was not the case where sleeping sickness did not occur. Further, it was found that trypanosomes from the cerebro-spinal fluid of sleeping sickness cases and from the blood of persons harbouring trypanosomes, but not suffering from disease symptoms, gave rise in monkeys to the same group of chronic effects which resembled the last stages of the disease in man. These facts led the Commissioners

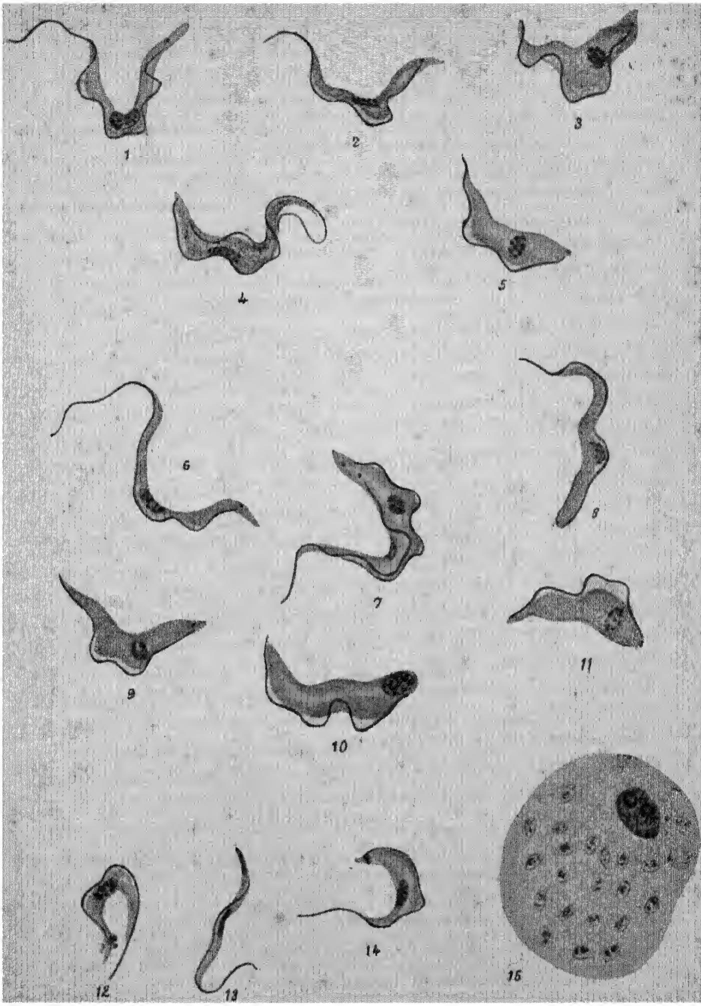


FIG. 187.[1]—Pathogenic Trypanosomes. × 1500.

1–5. *T. gambiense.*
6–11. *T. rhodesiense.*
12–15. *T. cruzi.*

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[1] For this figure, reproduced from *The Practice of Medicine in the Tropics*, we are indebted to Colonel Byam and Dr. Archibald and to Professor Warrington Yorke.

working on the Luangwa (a tributary of the Zambesi), that *Gl. morsitans* could transmit trypanosomes from human cases to rats, the cycle in the fly being at least eleven days, and that a definite percentage of wild flies in this region harboured the human parasite. There is thus no doubt that man, in widely extended regions of southern Central Africa, is exposed to danger when bitten by *Gl. morsitans*. As in the case of *T. gambiense* only a small proportion of flies fed upon an infected animal become infective. The infectivity of the trypanosome for the fly seems to depend in great measure on the species of the vertebrate host harbouring the trypanosomes on which the flies are fed (Corson).

It is now generally accepted that *T. rhodesiense* is a species distinct from *T. gambiense*. The disease in man tends to be more acute ; frequently there is not a terminal sleeping sickness stage, and there is less pronounced infection of lymphatic glands. In monkeys experimentally infected the trypanosomes multiply in the myocardium at a distance from the blood vessels and lead to myocarditis and pericarditis (Peruzzi). The organism is also more virulent for animals, the duration of the illness being shorter, and the susceptibility of the sheep and goat is greater than towards *T. gambiense*. In both of these animals widespread œdema, especially of the face, is a marked characteristic. *T. rhodesiense* infection in mice is influenced by human serum, which has no therapeutic effect on the infection with *T. gambiense* in these animals. The organism has been cultivated on Novy and MacNeal's medium. There has been considerable controversy regarding the relationship of *T. rhodesiense* to *T. brucei*. The lack of infectivity of *T. brucei* for the human subject has been repeatedly proved by the inoculation of volunteers. But while differences in the pathogenic effects of the two organisms have been observed, the right interpretation of the data constitutes a difficult question. Bruce and his co-workers, founding largely on extended biometric investigations, were of opinion that the *T. rhodesiense* is a strain of *T. brucei* which has adapted itself to man, and this view is now widely held.

Trypanocidal Action of Serum, etc.—Both human serum and the serum of infected animals have been found to possess trypanocidal and protective properties. A fact which is important in connection with the resistance of man to trypanosome infections is that while normal human serum has a marked trypanocidal action on various trypanosomes pathogenic to animals, e.g. *T. brucei*, *T. rhodesiense*, *T. congolense* and

T. equiperdum, it is without action on *T. gambiense*. Differences in their susceptibility to human serum, however, do not wholly account for the differences in infectivity for man. Collier rendered a strain of *T. brucei* resistant to human serum, but on inoculation it proved to be non-infective for human volunteers. Further, although baboons are resistant to *T. gambiense* inoculated into the blood stream, their serum is only weakly trypanocidal. It is of interest in connection with their natural immunity that Regendanz succeeded in producing a fatal infection of the central nervous system in baboons by intraspinal inoculation with *T. gambiense*.

In certain cases a degree of immunity has been established as the result of treating infections with trypanocidal compounds. It has not hitherto been possible, however, to produce such a degree of immunity as could be utilised either for prophylactic or therapeutic purposes. The serum of an infected animal may manifest a specific agglutination reaction towards the infecting trypanosome. Also, under the influence of the specific antibodies the homologous trypanosomes in a drop of citrated blood quickly become covered with blood platelets (adhesion phenomenon of Rieckenberg) or other small particles such as bacteria. A point of great importance, as bearing on the occurrence of relapses, is that brief contact *in vitro* of the antiserum with the parasites renders the latter serum-resistant. This is shown when the treated organisms are inoculated into animals rendered immune to the original strain; the infection develops in these as it would in a normal susceptible animal (Ehrlich, Roehl and Gulbransen). On account of this tendency of trypanosomes to alter in their behaviour to antisera, serological methods cannot be relied upon for distinguishing different species of those organisms.

Methods of Examination.—The organisms should be looked for in fresh films of the cerebro-spinal fluid, the blood, or the juice of glands. In the case of the *cerebro-spinal fluid*[1] about 10 c.c. should be centrifuged for fifteen minutes and the deposit placed under a cover-glass for examination; it is better to make a little cell on a slide by painting a ring of ordinary embedding paraffin, to place the droplet of fluid in its centre, and to support the cover-glass on the paraffin; in this way injury to the delicate structure of the organism is avoided. In fresh cerebro-spinal fluid the trypanosomes can be seen to be actively motile; the number in which they occur varies very much, and the same is true to a greater degree of the blood, in which they are, however, usually very scanty. With regard to the examination of the *blood*, Bruce

[1] Cerebro-spinal fluid may be obtained either by lumbar or cisternal puncture.

and Nabarro stated that it is difficult by ordinary centrifuging to concentrate the organisms, as these are not readily precipitated. They accordingly recommended that the blood be mixed with citrate of sodium solution (equal parts of blood and of a 1 per cent. citrate solution) and centrifuged for ten minutes, that the plasma be removed and centrifuged again for the same time, and that this be repeated three times, the deposit on each occasion after the first centrifuging being carefully examined. Greig and Gray have insisted that examination of the *lymph glands* in a suspected case forms the most ready means of arriving at a diagnosis, and this opinion has found strong support from the work of Dutton and Todd. The method is to puncture the gland with a hypodermic needle, suck up a little of the juice, and blow it out on to a slide. In all cases where stained films of any kind are to be prepared the methods of Leishman or Giemsa are to be recommended. Often in cerebro-spinal fluid and gland juice the staining of the chromatin is difficult, but good preparations are obtained by the procedure recommended by Leishman for studying the parasite in sections (p. 824). The presence of scanty parasites may be shown by *inoculation of a rat or mouse* with blood or cerebro-spinal fluid from a suspected case. The animal's blood should then be examined at intervals of several days for the next month or two. (This is readily done by snipping a small piece off the skin of the tip of the tail, and expressing a drop of blood.) It must be noted, however, that negative results have no significance, as an infection may fail to develop in the animal even when trypanosomes can be detected by microscopic examination in the inoculated blood. The occurrence of *auto-agglutination of the red corpuscles* when a wet film of blood of a patient is examined between a slide and coverslip is highly suggestive of trypanosomiasis.

Trypanosoma cruzi.—An important disease of man associated with trypanosome infection occurs in Brazil, where Chagas originally observed the trypanosome in a monkey, the intermediate host being the reduviid bug, *Triatoma megista* (*Conorhinus megistus*) (Fig. 187, 12–14). As these insects also feed on man both in their winged and nymph stages, the possible relationship of the trypanosome to a human disease in that region was investigated. This disease affects children, and gives rise to marked fever, the occurrence of œdema (conspicuous in the face), and enlargement of the thyroid, lymphatic glands, the spleen and liver. It may cause death in a few weeks, or assume a very chronic form characterised by disorders of internal secretion—myxœdema, bronzing of skin, and infantilism—although the relation of these changes to the infection has been doubted. The trypanosomes are readily found in the peripheral circulation in acute cases; but at the chronic stage they may not be detectable on microscopic examination, although they may persist for many years. The special feature of interest is the multiplication of the parasite, which does not

occur in the blood but in the tissue cells. Thus *post mortem* in man, the parasite is found chiefly within the cardiac and voluntary muscle cells and in the neuroglia of the central nervous system, in which situations there occur enormous collections of the organism in a Leishmania form, which is the stage of multiplication (Fig. 187, 15); finally the trypanosome form is regained and the parasites become free through rupture of the cell. Similar appearances are met with in infected animals. On account of this form of development of the parasite it has been called *Schizotrypanum*. The trypanosome is readily transmitted to a great variety of domestic and wild animals. As seen in the blood there are slender forms, which show active movements of progression, and larger forms which do not shift their place. As the parasite is very fragile it is damaged in making thin films of blood, and so for obtaining stained preparations thick films should be used, or citrated blood should be fixed with osmic acid vapour and films then spread. Cultures are obtainable on Novy and MacNeal's medium and retain their infectivity for many passages. A cycle of development takes place in the intestinal tube of the *Triatoma*, and the infection is transmitted through contamination of mucous membranes or of wounds in the skin by the fæces of the bug. Other reduviid bugs also carry the infection. The armadillo is probably the usual vertebrate host; but domestic animals, *e.g.* cats, have also been found to be infected. The trypanosome has been discovered to be much more widely distributed geographically than was originally observed and has been met with as far north as California in the rodent *Neotoma fuscipes*. Accordingly, the occurrence of the human disease appears to depend on a number of factors acting concurrently.

Diagnosis.—Where trypanosomes cannot be seen in the blood by microscopic examination their presence may be detected by injecting blood into a susceptible animal such as a guinea-pig. Brumpt's method of "xenodiagnosis" has found a valuable application, in which "clean" *Triatoma* or *Rhodnius* bugs reared in the laboratory are allowed to feed on the patient; the trypanosomes then develop in the insects. A complement-fixation method with the patient's serum and a glycerol extract of the heart and spleen of infected dogs, has been suggested.

Trypanosomiasis in Animals

Nagana or Tsetse Fly Disease.—This disease deserves special mention since, although it affects animals chiefly, there is little doubt that a proportion of cases, if not all, are due to a trypanosome (*T. rhodesiense*) which is capable of infecting man. Under natural conditions chiefly horses, cattle, and dogs are attacked; it is prevalent especially in certain regions of South Africa, though it

probably may occur elsewhere. In the horse the chief symptoms are the following: the animal is observed to be out of condition, its coat stares, it has a watery discharge from the eyes and nose, and the temperature is elevated; swellings appear on the under surface of the abdomen and in the legs; it gradually becomes extremely emaciated and anæmic, and dies after an illness of from two or three weeks to two or three months. In other animals the symptoms are of the same order, though the duration of the disease varies much; thus in the dog the illness does not last more than one or two weeks, while in cattle it may continue for six months. It is doubtful whether a domestic animal attacked by the disease ever recovers. The popular idea regarding the etiology of the disease was that it was contracted by animals passing through certain rather restricted and sharply defined areas or belts characterised by heat and damp, sometimes lying beside rivers, and always infested by the tsetse fly (*Glossina morsitans*), to the bite of which the disease was attributed. In this connection it is important to note that though man is frequently bitten by the tsetse fly he only rarely becomes infected with these trypanosomes. Modern knowledge on nagana dates from the discovery made by Bruce in 1894 that the blood of affected animals swarmed with trypanosomes. It was found that the parasite was present in the blood of every animal suffering from nagana and absent from the blood of healthy animals in the affected districts; further, the fever which marks the onset of the disease was shown to be accompanied by the appearance of trypanosomes in the blood; and finally, it was proved that the transference of a minute quantity of blood from an affected to a healthy animal originated the disease. As regards the part played by the tsetse fly in the condition, Bruce found that if flies taken from the fly belt, but which had not fed on an infected animal, were transported to a place where nagana did not occur, kept for a few days, and then allowed to bite susceptible animals, the latter did not contract the disease—this result showing that it was not, as had been supposed by some, a poison natural to the insect which was the pathogenic agent. But if such a fly was allowed to bite a dog suffering from the disease and then to bite a healthy dog, the latter contracted the malady and abundant trypanosomes were found in its blood. Again, threads dipped in the blood of an infected animal and allowed to dry caused the disease in healthy animals up to, but rarely beyond, twenty-four hours after being dried. If, however, the blood were kept moist, then it retained its infectiveness up to between four and seven days; up to forty-six hours living trypanosomes could be seen in the tube of the fly's proboscis. Further, Bruce showed that infection did not occur by any food or water partaken of by an animal while going through a fly belt, for he took horses through such a region without allowing them to eat or drink, and found that they still contracted the infection, if during their few hours' journey through the belt they had been bitten by the tsetse fly. Finally, he showed that if flies were taken from an infected area to a healthy one a few miles off and allowed to bite healthy animals, the latter contracted nagana.

By those experiments it was thus determined that nagana could be transmitted by the blood of the infected animal—that is, without the agency of the fly; that the latter had no inherent power to

produce the disease; that it could, however, by successively biting infected and healthy animals, transmit the disease to the latter; and that specimens of the insect caught in infected areas harboured the parasite and were thus infective The question remained as to how the flies might become infected in nature. It had been observed that in districts where the tsetse fly lived, the prevalence of the disease in imported animals was related to the presence in the locality of wild herbivora. Bruce now found that, if considerable amounts of the blood of the latter were taken to another

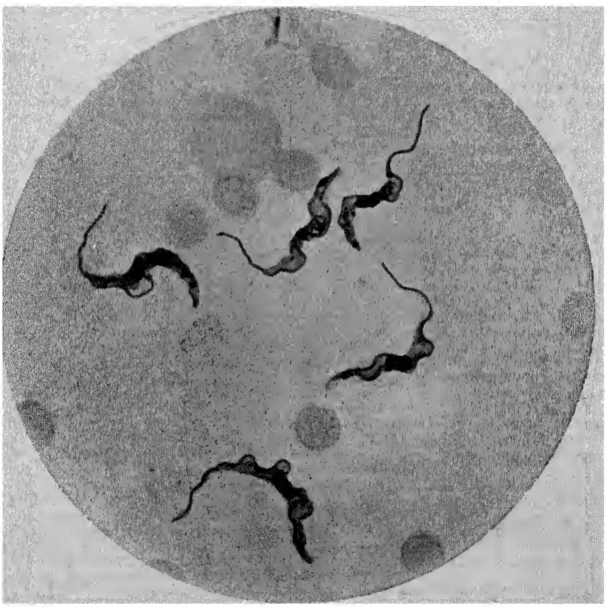


FIG. 188.—*Trypanosoma brucei* from blood of infected rat. ×1000.

locality and injected into dogs, these in a proportion of cases contracted nagana, and from this it was deduced that the wild animals harboured the parasites in small numbers in their blood and so acted as a reservoir of infection. Bruce's work as a whole pointed to the trypanosome as the cause of nagana, and this has since been finally established by producing the disease through the agency of flies bred under laboratory conditions and experimentally infected, as well as by artificial cultures of the organism. The trypanosome undergoes a cyclic development in the body of the insect similar to that of *T. gambiense*.

The trypanosome originally recovered by Bruce was investigated as regards its microscopic characters by Plimmer and Bradford,

who named it *T. brucei* (Fig. 186). It was found later to differ in its morphology from the trypanosome found subsequently by Bruce and others to be commonly associated with the disease nagana, the latter having the characters of *T. rhodesiense*, whereas the original *T. brucei* is monomorphic and resembles *T. evansi* (Stephens and Blacklock). It remains unsettled as to whether these differences indicate specific characters or whether the original *T. brucei* became altered as the result of prolonged sojourn in laboratory animals. Novy and MacNeal succeeded in cultivating this trypanosome, though it was very difficult to obtain a first growth from the blood on their blood agar medium ; once started, however, it was maintained through many subcultures, the optimum temperature of growth being 25° C., and it was from these subcultures that the infection was obtained which definitely proved the organism to be the cause of the disease.

Nearly all laboratory animals are susceptible to infection, and the duration of the illness corresponds to what has been observed in the natural infection of these animals. But repeated passage through a particular species tends to increase the virulence of the trypanosomes for that species. Thus *T. brucei* when accommodated to mice leads to death of these animals with septicæmia in three to four days after inoculation, and in rats the result is similar. Rabbits are highly susceptible to artificial infection with *T. brucei* ; in these animals the disease pursues a chronic course and they may live for several months.

Serious disease in cattle, horses, sheep, and other domestic animals in Africa is caused also by *T. congolense* and *T. vivax*. *T. congolense* is a small monomorphic trypanosome devoid of flagellum which measures 9–18 μ by under 3 μ. It is transmitted by *Gl. morsitans* and other tsetse flies. This trypanosome does not invade the salivary gland of the fly, but passes from the stomach to the labrum. *T. vivax* is distinguished by its active movements of progression ; it measures 18–26 μ in length, has a definite flagellum, and the macronucleus lies toward the anterior end of the body. In the tsetse fly (*Gl. morsitans* and other species) multiplication of the trypanosomes occurs only in the proboscis.

It is beyond the scope of this work to deal at length with the other trypanosome infections of animals, but it may be said that many species have been described in various mammals, birds, and fishes, and that these are spread either by insects or by leeches. One of the most interesting is Dourine, a condition resembling in many ways nagana, caused by *T. equiperdum*. It, however, presents this peculiarity, that infection does not take place by an intermediate host, but apparently directly through coitus, as it occurs only in stallions and in mares served by these. Surra is also due to a trypanosome (*T. evansi*) ; although first observed in horses in India, the infection is widely spread in Asia, Africa, and the Philippines, and severely affects domestic animals and camels. The infection appears to be spread directly by biting flies (various species of Stomoxys and Tabanus). Mal de Caderas, a disease of horses in South America, is also due to a trypanosome (*T. equinum*). Biting flies are believed to transmit this infection also. The parasites of dourine and surra resemble the slender forms of *T. gambiense*. *T. equinum* is similar in size, but in it there is only a minute blepharoplast and the parabasal body is

absent. The trypanosome found in cattle in Africa and other parts of the world (*T. theileri*) is of doubtful pathogenicity. *T. melophagium*, a non-pathogenic trypanosome of wide occurrence in sheep, is transmitted by the sheep-ked, in the hind-gut of which the infective forms of the parasite develop.

Trypanosoma lewisi.—This trypanosome is very common in the blood of rats all over the world, though the percentage of animals affected varies in different localities. The organism has no importance from the standpoint of human pathology,[1] but it is of great interest that the infection runs a very definite course in the rat, although it is very rarely fatal. A fatal issue may, however, occur in young individuals, especially when these are infected with strains of the organism imported from other localities. The trypanosome, as it appears in the blood in the later stage of the infection, is actively motile, is of ordinary length but is somewhat narrow, the posterior end is pointed, the macronucleus lies in front of the centre of the body, and the protoplasm does not contain any granules. It multiplies by fission, of which Laveran describes two varieties In one, the organism splits longitudinally and gives rise to smaller individuals than the parent In the other, the trypanosome loses its ordinary shape and becomes more oval: nuclear division, which is often multiple, then takes place, and on subsequent division of the protoplasm a number of small flagellate organisms result; these last may attain the full form and size before dividing again, or they may divide when still small. When a rat is infected by injection into the peritoneum, active multiplication goes on in the cavity for a few days and then comes to an end. Very soon after infection the organisms begin to appear in the blood, and there rapid multiplication occurs, the extent of which is sometimes so great that the trypanosomes may seem to equal the red blood corpuscles in number. The animal usually shows no symptoms of illness. The infection goes on for about two months, and then the organisms gradually disappear from the blood. In the great majority of cases the rat is now immune against fresh infection. If trypanosomes be introduced into its peritoneum they are, according to Laveran, taken up by mononuclear phagocytes and destroyed. The serum of a rat which has been infected shows agglutinating action on the trypanosomes, causing them to agglomerate in rosettes in which the flagella are directed outwards; the serum of immune rats has also a certain degree of protective action if injected along with the organisms into a susceptible animal. As has already been noted, this trypanosome has been cultivated on artificial media, on which it multiplies freely, large numbers of small forms being often produced (the optimum temperature is about 20° C.). These when injected into rats give rise to the usual infection. Novy and MacNeal succeeded in carrying a growth through many subcultures. The trypanosome is very resistant to cooling, and has been exposed for fifteen minutes to the temperature of liquid air (—191° C.) without being killed. Rabinowitsch and Kempner have shown that the rat flea, *Ceratophyllus fasciatus*, transmits the parasite, infection

[1] Johnson has recorded the presence transiently of trypanosomes morphologically indistinguishable from *T. lewisi* in the blood of a native infant in India. (*Trans. Roy. Soc. Trop. Med. Hyg.*, 1932–33, xxvi. 467.)

occurring through the fleas or their fæces being swallowed (Nöller, Minchin and Thomson, and others). In the flea a phase of multiplication of the trypanosomes occurs within the epithelial cells of the stomach; the parasites then migrate to the hind-gut and rectum, assume the crithidial form and undergo fission, and finally take on the trypanosome form again. The flea becomes infective about a week after biting, and remains infective for the rest of its life. Infection may also take place through other species of fleas and through the rat louse (*Hæmatopinus spinulosus*).

LEISHMANIASIS

Under this term there are grouped three human diseases caused by protozoal parasites whose exact zoological place is not yet definitely settled. These organisms are the *Leishmania donovani*, associated with the human disease kala-azar; *Leishmania infantum*, derived from a similar disease occurring in children; and *Leishmania tropica*, which has been found in a skin ulceration of widespread geographical distribution. Microscopically the organisms are practically identical, but it is probable that they belong to two, or possibly three, distinct species. A similar parasite is associated with a disease in dogs, the symptoms of which in many respects resemble those met with in the human subject. The geographical distribution of canine leishmaniasis partly corresponds with that of the human varieties, but the association of leishmania infections in the dog and the human subject is by no means invariable.

Leishmania donovani.—Leishman noticed in several soldiers invalided from India for remittent fever and cachexia that very careful examination of the blood failed to reveal the presence of the malarial parasite. Most of these patients had been quartered during their service at Dum-Dum, an unhealthy cantonment near Calcutta, and from this fact the existence of a disease hitherto undescribed was suspected. In 1900 Leishman found in the spleen of such a case peculiar bodies, which resembled degenerating forms of *T. brucei*, and he suggested that they might be trypanosomes. Leishman's observations were confirmed in India by Donovan, and the bodies associated with the disease are now usually called the "Leishman" or the "Leishman-Donovan" bodies. They were found by Bentley, and later by Rogers, in the disease known in Assam as kala-azar, the pathology of which had long been obscure, since, while it resembled malaria in many ways, no malarial parasites could be demonstrated. This disease has gone under various synonyms, *e.g.* cachectic fever, Dum-Dum fever, non-malarial remittent fever, but is now recognised as a single specific entity.

Kala-azar (or "black disease,"—so called from the hue assumed by chocolate-coloured patients suffering from it) has been known since 1869 as a serious epidemic disease in Assam, where it has spread from village to village up the Brahmaputra valley. The disease is now known to occur in various sub-tropical centres—cases where the Leishman bodies have been found having been met with in many parts of India, China, Turkestan, the Malay Archipelago, North Africa, the Soudan, Syria, and Arabia. The disease is characterised by fever of a very irregular type, by progressive cachexia, and by anæmia associated with enlargement of the spleen and liver. Some cases at the commencement may resemble enteric fever. Rogers has pointed out that there occurs a leucopenia which differs from that of malaria in that it is almost always more marked —the leucocytes usually numbering less than 2000—and further, in that the white cells are always reduced in greater ratio than the red corpuscles, which condition, again, does not occur in malaria. The disease is chronic, often going on for several years, and in, at any rate, 80 per cent. of the cases has a fatal issue. It is of interest, in connection with the cutaneous disease caused by *L. tropica* (*vide infra*), that in kala-azar ulcers of the skin may occur. Also, Acton and Napier have recorded the development of skin nodules, which contained the parasites, as a late form of recurrence in patients who had not been completely sterilised by treatment.

Post mortem, there is little to note beyond the enlargement of the liver and spleen, but in the intestine, especially in the colon, there are often large or small ulcers, and there is evidence of proliferation in the bone marrow, the red marrow encroaching on the yellow.

In a film made from the spleen and stained by Leishman's stain, the characteristic bodies can be readily demonstrated (Fig. 189). They are round, oval, or, as Christophers has pointed out, cockle-shell-shaped, and usually 2·5 to 3·5 μ in diameter, though smaller forms occur. The protoplasm stains pink, or sometimes slightly bluish, and contains two bodies taking on the bright red colour of nuclear matter when stained by the Romanowsky combination. The larger (trophonucleus), which stains less intensely than the smaller, is round, oval, heart-shaped, or bilobed, and lies rather towards the periphery of the body—in the region of the "hinge" in the cockle-shaped individuals. The other chromatin body (kinetonucleus or kinetoplast) is usually rod-shaped, and is set perpendicularly or at a tangent to the larger mass, with which only exceptionally it

appears to be connected. According to Christophers, Shortt and Barraud, the smaller chromatin body is differentiated into the parabasal body, and, running at right angles to the long axis of the latter, the rhizoplast (axoneme), which is a straight or slightly curved linear structure measuring about half the diameter of the parasite in length. The aspect of the parasite on which the parabasal body is situated is taken to be dorsal, and from it, at a point corresponding to the blepharoplast, the rhizoplast extends anteriorly. Usually the protoplasm contains one or two vacuoles. Though in spleen smears many free bodies are seen, the study of sections shows that ordinarily their position is intracellular, — the cells containing them being of a large mononuclear type (Fig. 189). The view held is that on their entering the circulation they are taken up by the mononuclear leucocytes and by such cells as the endothelial lining of the splenic sinuses or those lining capillaries or lymphatics, that in these cells multiplication takes place,—it may be to such an extent as to rupture the cell, — and that if thus the bodies become free they are taken up by other cells and the process is repeated. The clusters of bodies sometimes seen in smears are probably held together by the remains of ruptured phagocytes. In capillaries the endothelial cells after ingesting the bodies probably become detached from the capillary wall, as they are often observed free in the lumen of the vessel—this being well seen in the hepatic capillaries. The parasites are found in greatest abundance in the spleen, liver, and bone marrow, and also in mesenteric glands, especially in those draining one of the intestinal ulcers ; less frequently they occur in the skin ulcers, and in other parts of the body, *e.g.* the villi of the intestine may be crowded with them (Perry). Donovan described them as occurring in the peripheral blood, especially within the leuco-

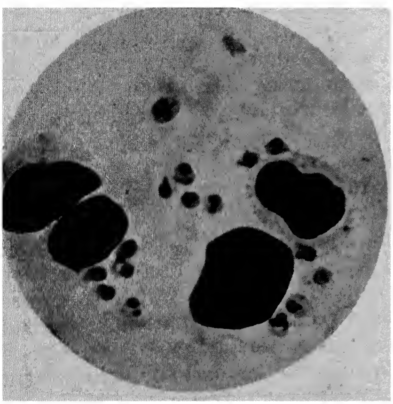


Fig. 189.—Leishman-Donovan bodies from spleen smear ; some are within the endothelial cells. Leishman's stain. ×1000. See also Plate VI., Fig. 25.

cytes, and this observation has been generally confirmed, though sometimes prolonged search is necessary. Patton has found that the numbers in the blood vary from time to time, and special incursions may be associated with exacerbations of dysenteric symptoms which he holds indicate a spread of the intestinal ulceration. Shortt and his co-workers have found that the organisms can be frequently recovered in cultures made from the urinary sediment.

In the body the parasite multiplies by simple fission, both nuclei dividing amitotically, and two new individuals being formed; but sometimes a multiple division takes place, each nucleus dividing several times within the protoplasm and a corresponding number of new parasites resulting.

FIG. 190.—*Leishmania donovani.* Leptomonas form from culture. Leishman's stain. ×1000.

In view of Leishman's original opinion, an extremely important discovery was made by Rogers, to the effect that in cultures a flagellate organism developed from the Leishman-Donovan body. Cultivation was effected by taking spleen juice containing the parasite, placing it in sodium citrate solution, and keeping it at 17° to 24° C. Under such conditions there occurs an enlargement of the organism, but especially of the larger nucleus. This is followed by the appearance of a pink-staining vacuole in the neighbourhood of the smaller nucleus. Along with these changes, in from twenty-four to forty-eight hours the parasite becomes elongated and the smaller nucleus and its vacuole move to one end; from the vacuole there then appears to develop a red-staining flagellum, which when fully formed seems to take its origin from the neighbourhood of the small nucleus. The body of the parasite is now from 20 to 22 μ long and 3 to 4 μ broad, with the flagellum about 22 μ long. The whole development occupies about ninety-six hours. The formation of an undulating membrane was not observed, and, although the flagellated organism moved flagellum first, like a trypanosome, it is evident that here the relationship of the micronucleus is different, as this structure lies anterior to the macronucleus. The serum of many animals, *e.g.* man, guinea-

pig, has an inhibitory effect on the parasite, but success in the cultivation has attended the use of Novy and MacNeal's medium made up with rabbit's blood (that of the sheep or dog may also be used).[1] The phases of the parasite in such cultures have been investigated by Christophers, Shortt and Barraud; they concluded that the masses of mature flagellate forms which develop from the Leishman-Donovan bodies leave the group where they were produced and become free-swimming. These separate and later come to rest; then as a result of repeated divisions each gives rise to a number of short multiplicative forms, some of which again are provided with vibratile flagella and, becoming free-swimming, repeat the cycle. In cultures which have been kept some time (at least seven days), small rounded non-flagellate forms appear which measure about 2 μ; these are considered by Row to be of the nature of cysts, but have been regarded by others as degenerate forms. In old cultures Leishman described the occurrence of unequal division of the parasite, which resulted in the splitting off of a hair-like undulating form containing a chromatin granule; the final development of these spirillary forms has not been traced.

As regards the classification of this organism, which now usually goes by the name *Leishmania donovani*, given to it by Ross, although its flagellated form is that of a *leptomonas*[2] (Fig. 190), and so differs from the typical trypanosome form, it bears considerable resemblance to the members of this group. But as Leishman has pointed out, the cultures may not represent the full development of the organism in the trypanosome direction. Minchin's suggestion has been accepted, however, that in the present incomplete state of knowledge it is well to place it and its congeners in a provisional genus, *Leishmania*, of the Flagellata.

Experimental Infections.—Though results obtained in different parts of the world vary somewhat, certain animals (*e.g.* monkeys, young dogs, and mice) have, in a varying proportion of cases, been infected with the parasite as it occurs in human lesions

[1] The following procedure has been recommended as improving the medium for cultivation of leishmaniæ. To each tube of 18 mm. diameter containing 9 c.c. 2 per cent. melted agar medium 3 c.c. defibrinated rabbit blood are added, and the tubes are kept in the sloped position at room temperature for twenty-four hours. They are then capped and placed at 37° C. for twenty-four hours. Thereafter 2 c.c. 0·85 per cent. NaCl solution are added to each tube and they are capped again and placed in a sloping position at 37° C. for twenty-four hours further.

[2] In the leptomonas the blepharoplast is situated at the anterior end, and there is no undulating membrane.

and also in cultures. The intraperitoneal route is the best, and both when the animals have died and have been killed leishmaniæ have been found in such situations as the spleen, the liver, and bone marrow. Recovery from the infection is followed by immunity to re-inoculation. Feeding experiments have usually been unsuccessful, but one or two positive results are recorded. In India the examination of dogs from the neighbourhood of kala-azar cases has not yielded evidence that natural infection occurs in these animals. The observation of Smyly and Young that the Chinese hamster (*Cricetulus griseus*) and others are highly susceptible to intraperitoneal inoculation has greatly facilitated such investigations. Infection has also been produced, in descending order of certainty, by the subcutaneous, oral, percutaneous, and conjunctival routes. Brumpt and Gaillard have found the spermophile (*Citillus citillus*) susceptible in the case of a Chinese strain of the parasite.

The question arises, given that the *Leishmania donovani* is the cause of kala-azar, how is infection spread ? Various theories have been held, *e.g.* that ingestion of the parasite produced infection, but the extensive work of the Indian Kala-azar Commission with mice lent no support to this view. The relationship of the organism to the trypanosomes suggested that an insect might act as the intermediary host, and the bed-bug (*Cimex rotundatus*) was suspected, but it was shown that bugs obtained from the bedding of kala-azar patients, or fed upon such cases, very rarely contained flagellates, and material from such bugs when injected into monkeys did not produce the infection. As regards other insects, a frequent difficulty has been the presence of a variety of flagellates which normally inhabit their intestines. The Kala-azar Commission, however, brought forward strong evidence that a sandfly, *Phlebotomus argentipes*, is the insect host. When flies of this species were fed upon cases of kala-azar, flagellates appeared in the intestine of 43 per cent. (Napier and Smith). In view of the scantiness of leishmaniæ in the peripheral blood of patients, such multiplication in this fly indicates that it affords a specially favourable medium for the parasite. The appearances met with in the intestinal contents of the infected flies were the same as those found in artificial cultures of leishmaniæ; further, a large number of flies fed upon healthy individuals failed to show flagellates. It is of importance also that in several instances the Commission carried out control experiments in which the same cases as caused infection of *P. argentipes* were fed upon

by bugs, but with entirely negative results. Infected sandflies showed a marked infection of the gut five days after feeding upon a case, and in a considerable proportion of flies which survived to the eighth day the pharynx and buccal cavity contained flagellates, but they did not become established to any extent in the rectum. In connection with the identification of insect hosts of the leishmaniæ, it is noteworthy that these protozoa will develop only in the absence of bacteria; this condition is realised in the alimentary tract of phlebotomus flies. As regards capacity to transmit the disease, it is only in certain species of these flies that the proliferated leishmaniæ make their way to the proboscis, whence apparently the parasites are conveyed to the human host by the fly in the act of biting. The conclusive experiment of transmission of the infection, to the Chinese hamster, by repeated bites of artificially infected *Phlebotomus argentipes* has been reported by Shortt (1931) and by Napier, Smith and Krishnan, the animals dying fourteen to sixteen months after inoculation. Complete development of the parasite occurs also in *P. sinensis*. It is, of course, not excluded that other blood-sucking insects may also carry the infection in different parts of the world.

Methods of Examination.—The Leishman-Donovan bodies can be readily seen in films or sections of the infected organs. These should be stained by the Romanowsky stains. Fluid taken from the enlarged spleen with a perfectly dry needle during life may be examined, but it is probable that in this disease puncture of the spleen may not be a very safe operation, as death from hæmorrhage from this organ is not uncommon; liver-puncture is less dangerous, but the parasites are not so readily found in this way; the juice of enlarged lymphatic glands may also be examined. The microscopic demonstration of the parasite in the circulating blood should always be attempted by means of films made on slides; the thick edge should be examined specially, since the parasites are usually contained in the leucocytes which accumulate at this part of the film. Another method consists in inoculating a number of tubes of Novy and MacNeal's (N.N.N.) medium each with several drops of blood from the patient; or in adding 10 drops of blood aseptically to 10 c.c. citrate and salt solution (p. 144), sedimenting the corpuscles, and then by means of a pipette inoculating tubes of the medium with the sediment. The cultures should be kept at 22° to 26° C. and examined after a few days, and again after two or three weeks. The absence of malarial parasites from the blood and the leucopenia which has been alluded to are also

important features. Napier's "aldehyde reaction" consists in the addition of one drop of formalin to 1 c.c. of blood serum with thorough mixing; when the serum is derived from a fully developed case of kala-azar the mixture in a few minutes resembles boiled egg-white. This result is said to be obtained with serum from no other condition, except possibly schistosomiasis. A similar reaction has been got with a pentavalent antimony compound as the reagent.

Leishmania infantum.—Nicolle, working in Tunis, observed a disease clinically identical with kala-azar, usually affecting children between two and five years of age, and exceptional after the age of fifteen. He found in the spleen, liver, and bone marrow in such cases an organism microscopically indistinguishable from the *Leishmania donovani*. Cutaneous and intestinal ulceration, such as are met with in kala-azar, do not occur. The disease, although sporadic, is very widespread, and occurs along the whole of the south and east littorals of the Mediterranean, in Spain, Portugal, Greece, Sicily, France (Côte d'Azur), and in Italy as far north as Rome, in the Soudan and Abyssinia.

The organism can be successfully inoculated in the dog, monkey, mouse, rat, guinea-pig and rabbit by intrahepatic and intraperitoneal injection of spleen pulp from fatal human cases. The organism can be cultivated on a modified form of Novy and MacNeal's medium (p. 827), cultures presenting characters similar to those in the other leishmaniæ; and Novy and MacNeal have produced the disease by inoculation with massive doses of cultures. Several species of hamsters have proved to be susceptible to inoculation with the organisms from tissues or cultures (Adler and Theodor; and others).

Nicolle regarded the organism as a separate species to which he gave the name *Leishmania infantum*, and this is supported by the fact that the disease, as it occurs in the regions named, is apparently confined to young children. He considered the infection of the dog to be significant, as this animal might be the channel through which children become infected, for in most regions where the disease prevails there occurs a disease of dogs, which may be either of an acute or chronic character, and which is apparently due to an identical organism. In infected dogs ulcers may occur in the skin and both in these and also in apparently healthy areas numerous leishmaniæ are present. Laveran found that a *Macacus* monkey which had recovered from an infection with leishmania derived from Tunis was also immune to inoculation with parasites obtained from a case of Indian kala-azar; thus it would appear likely that the

two organisms are identical, although they show differences in virulence and resistance. Serological evidence is also in favour of their identity (p. 854). The evidence points to *Phlebotomus perniciosus* being the important vector (Adler and Theodor). But other phlebotomus flies may also be vectors, e.g. *P. major*. Inoculation is effected by regurgitation of the parasites from the fly at the time of biting.

Leishmania tropica.—In various tropical and sub-tropical regions (India and other parts of the East, Egypt, Northern Africa, Southern Russia, Turkey, South America, West Indies) there is widely prevalent a variety of very intractable chronic ulceration which goes by various names in different parts of the world — Delhi sore, tropical ulcer, Aleppo boil, etc. These sores may spread from one part of the body to another by auto-inoculation, also they may be communicated to other individuals by contact. The work of J. H. Wright first showed that a protozoal parasite is concerned in the etiology of the condition. In the discharge from the ulcer and in sections of a portion of tissue excised from a case coming from Armenia, Wright observed great numbers of bodies which are indistinguishable morphologically from the Leishman-Donovan body. It was found that the bodies were usually intracellular in position in the lesion, as many as twenty being in one cell, and that the type of cell containing them was as in kala-azar, that derivable from endothelial tissues (Fig. 191).

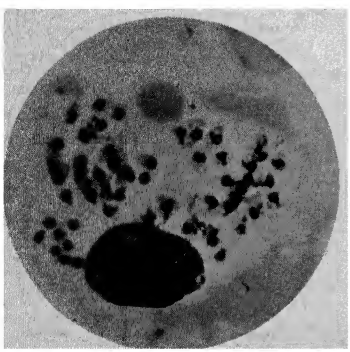


FIG. 191.—*Leishmania tropica.* Smear from sore showing endothelial cell containing numerous leishmaniæ bodies. Leishman's stain. ×1000.

Wright's observations have been fully confirmed by workers in various parts of the world, and it is now recognised that in these tropical ulcers we have another example of the activity of a leishmania. The duration of the sore is about a year. It is stated that after recovery the individual possesses immunity. Sometimes the parasite is destroyed in a foul ulcer, but can still be obtained by taking some of the juice from the marginal indurated tissues by capillary glass tubes. Patton reported having found the organism in blood taken from parts adjacent to

the ulcer. The organism may be grown in Novy and MacNeal's (N.N.N.) medium. Row has obtained cultures in citrated blood. Nicolle and Manceaux reproduced the condition in man, the monkey, and the dog, both by material obtained from the natural infection and by cultures on Novy and MacNeal's medium. The lesions were identical with those naturally occurring, the incubation period being often many months. The organism may still be pathogenic for man after cultivation for four and a half years. In the male mouse intraperitoneal injection is followed either by a granuloma in the testicle, or by a generalised infection in which lesions, often characterised by widespread destruction of tissues, occur in the skin or around the joints; all these lesions contain numerous parasites. Evidence has been furnished by Adler and Theodor in Palestine that the disease is transmitted by phlebotomus flies. They inoculated into the skin in human subjects the contents of the alimentary tract of *P. papatasii* which had been fed on lesions at least eight days previously and contained numerous leptomonas forms; from seventeen to thirty-three days later in a third of those inoculated a small papule developed at the site of inoculation, which contained leishmaniæ. *P. sergenti* may also act as the vector. Thompson and Balfour have described in the Soudan a condition in which subcutaneous nodules without ulceration occurred in man, and these contained leishmania bodies. Cutaneous leishmaniasis occurs in dogs, and Adler and Theodor have produced a typical skin lesion in the human subject by inoculation with a culture of canine origin.

In the South American form of the disease, which occurs especially in forest areas, after the skin ulcers have appeared, typical lesions occur not uncommonly at a later date in the nasal and buccal mucous membranes; such cases are liable to develop a severe cachexia. It has been suggested that this condition is due to a specific parasite, *L. braziliensis*. Dogs are affected as well as man. *Phlebotomus intermedius* is probably a vector.

Differentiation of the Leishmaniæ.—The close similarities in the morphology and effects of the three leishmaniæ naturally raise the question whether we are not dealing with variants of one organism whose differences depend on differences in the virulence of different types or on the susceptibility of different hosts. The following are some of the facts bearing on this question. The Indian and Mediterranean diseases are apparently identical clinically, although the age incidence is different. But in certain parts of India kala-azar is chiefly found in children below the age of fifteen, while on the other hand cases

occur in young adults in regions where the infantile variety prevails. The importance of such factors as racial susceptibility is indicated by the fact that in Tunis it is chiefly the children of Italian parentage who suffer. Kala-azar and Oriental sore are linked by the occurrence in the former from time to time of skin ulcers, although in these, unlike the case of Oriental sore, the parasites are difficult to find; on the other hand, it is of importance that cases of Oriental sore do not develop into kala-azar. But in dogs inoculation with *L. donovani* may cause skin lesions resembling those caused by *L. tropica* in man (Caminopetros). Again, Nicolle found that dogs infected with *Leishmania tropica* appeared to be less susceptible to *Leishmania infantum* than usual. The incidence of canine leishmaniasis in communities where a human infection prevails varies in different regions, *e.g.*, even in the Mediterranean littoral, though it is usually common where *Leishmania infantum* is found. But dogs are more extensively infected in Turkestan where the human incidence of the disease is among adults. The problem of the relationships of the leishmaniæ has been investigated by serological methods (Noguchi; Kligler; Adler and Theodor). It has been found that antisera obtained from rabbits by injecting intravenously cultures of the organisms possess both agglutinating and lytic action. The results obtained by the use of such sera indicate that *L. infantum*, *L. tropica*, and *L. braziliensis*, are specific organisms. On the other hand, *L. donovani* amd *L. infantum* are indistinguishable. But there appears to be a certain amount of group reaction among these organisms.

PIROPLASMS

Up to the present no human disease has been proved to be associated with the presence of piroplasms. But several important diseases of the lower animals are caused by protozoal parasites of this group, and a short account of the organisms may be given.

The piroplasms are pear-shaped unicellular organisms which vary in size according to the species from 3–4 μ in length down to less than 1 μ. The peripheral part is denser than the central, which often appears as if vacuolated, and at the broad end there is a well-staining chromatin mass. Sometimes irregular and ring-, rod-, or oval-shaped individuals occur. The organisms are found within the red blood corpuscles of the infected animal and also free in the blood. In the former situation there is sometimes only one within a cell, but the numbers vary under different circumstances and in different species. No pigment is formed. Multiplication takes place by fission, and the new individuals, two or four in number, remaining for longer or shorter times in apposition, account for some of the appearances seen in cells. Especially in the forms free in the blood, pseudopodial prolongations of the

protoplasm, usually from the pointed end, are developed, and it may be by means of such pseudopodia that entrance to the red cells is obtained. Infection is usually carried from infected animals by means of ticks, as Smith and Kilborne showed. In one case Koch described in the stomach of the tick, the development by the piroplasm of spiked protoplasmic processes which sprouted out from the broad end of the organism, and the occurrence of conjugation of two such individuals by their narrow ends to form a zygote. Observations by Christophers indicate that a globular body now appears, probably corresponding to the oöcyst stage of other similar protozoa, and the further development consists in a division into sporoblasts which may infect the whole tissues of the tick, especially the salivary apparatus. The eggs may also be infected and the young ticks developed from these may thus be capable of carrying the disease to fresh hosts (Smith and Kilborne). At first, however, the bites of the young ticks do not convey the infection, and it is only after several weeks that they become infective; this suggests that the parasite undergoes a cycle of development. Frequently when an animal has passed through an attack of piroplasmosis it is immune to the disease, and with regard to this immunity in certain cases very interesting facts have been observed. For instance, the condition may not be associated with the disappearance of the parasite from the blood of the immune animal, and the latter may thus be a source of danger to other non-immune animals with which ticks harboured by it may come in contact.

The following are some of the chief piroplasms causing disease in animals: *Piroplasma bigeminum* (*Babesia bigemina*). This was first accurately described by Theobald Smith, and is the cause of Texas or red-water fever, a febrile condition associated with hæmoglobinuria, which occurs in the Southern States of America, South America, South and Central Africa, Algeria, various parts of Northern Europe, and in Australia. The organism gets its name of *bigeminum* from the fact that it is often present in the red cells in pairs, which may be attached to one another by a fine thread of protoplasm; this probably results from the complete separation of two individuals being delayed after division has occurred. It is the largest of the piroplasms found in cattle. Infection is spread by the tick *Boöphilus bovis* (*Margaropus annulatus*), and some of the characteristics of the disease epidemiologically are explained by the fact that this insect goes through all its moultings on the same individual host; but other ticks have also been shown to spread the infection. *Babesia bovis* is also a cause of red water in cattle; it is much smaller than *B. bigemina*. The tick *Ixodes ricinus* transmits the infection. *Babesia equi* gives rise to biliary fever in horses in South Africa. As was shown by Theiler, it is carried by the tick *Rhipicephalus evertsi*. Mules and donkeys can also be infected. Young horses are less severely affected than older ones; after clinical recovery the blood of such "salted" animals can be used for the protective inoculation of young horses. *Babesia canis* causes a piroplasmosis occurring in dogs. The organism has been cultivated by Thomson and Fantham and others by the method of Bass and Johns (p. 803).

Piroplasma parvum (*Theileria parva*).—This organism was discovered by Theiler in the blood of cattle suffering from African East Coast fever, a serious disease characterised by fever and enlargement of

the lymphatic glands, but without the occurrence of hæmoglobinuria. The organism is small, appearing as ovoid or rod-shaped forms which measure 1–3 μ in length and 0·5–0·7 μ in breadth. *Rhipicephalus appendiculatus* and certain other ticks convey the disease. It is of interest that in the case of this organism, as contrasted with the other piroplasms described above, division occurs not in the red corpuscles but in endothelial cells of the lymphatic glands, spleen, and other organs. The schizonts appear as masses of protoplasm 3–10 μ in diameter, containing a large number of minute chromatin dots. The intracorpuscular forms of the parasite appear to be adapted for development in the tick, since inoculation of blood from an infected animal into other cattle does not produce the disease. With regard to the pathology of infection by piroplasms we know nothing. The diseases are often extremely fatal, carrying off nearly every individual attacked, the nature of the changes originated being unknown.

Bartonella Infections : Oroya Fever

This disease, which occurs in Peru, is characterised by fever of intermittent type along with severe rapidly progressive anæmia, and is fatal in a large proportion of cases. Although the causal agent now appears to be bacterial in nature, the condition may be considered here, since, as Barton found, the infective agents invade the red corpuscles. They are seen as minute coccal forms or as straight or slightly curved rods, measuring from 0·3 to 2·5 μ, which are motile in the fresh state ; when stained with the Romanowsky stains they have a reddish violet colour, the extremities being more intensely stained and often appearing thickened. A large proportion of the red cells may be affected. Strong and his co-workers of the American Commission gave the name of *Bartonella bacilliformis* to these bodies, which were considered to be probably protozoal and similar to *Theileria parva*. *Post mortem*, large collections of granules have been found in the vascular endothelial cells of the internal organs and especially of the lymph glands, which have been supposed to represent a stage in the multiplication of the parasite. Cultures of the organism on solid or semi-solid media have been obtained by Noguchi and Battistini after inoculation with citrated blood from a case of the disease. The organism possessed considerable resistance, as it survived in the blood for forty-three days. Cultures grew in leptospira medium (p. 693) and on sloped blood agar with a reaction corresponding to pH 7·8, but not on ordinary agar or in broth. The most suitable temperature for growth was found to be 25°–28° C. The organism as seen in cultures resembles the forms met with in the blood but is more pleomorphous and tends to form large clumps. It is Gram-negative and an obligate aerobe. In certain conditions it is motile, and flagella can be demonstrated by appropriate staining. Intravenous inoculation of pure cultures into young *Macacus rhesus* monkeys produced intermittent fever of long duration, the characteristic organisms appearing in scanty numbers in the red cells ; but anæmia did not occur in these animals. Passages were effected from animal to animal, and the organism was recovered from the blood, lymph glands, and spleen. Filtered cultures were inactive. After intracutaneous inoculation into the eyebrows in young *rhesus* monkeys,

vascular nodules developed which showed large numbers of the organisms situated within endothelial cells. These lesions are considered to be identical with those of verruga peruviana, a disease characterised by a nodular skin eruption. Also Noguchi isolated from verruga lesions an organism which was morphologically and serologically identical with that found in Oroya fever and which had similar pathogenic action. Further, monkeys which had recovered from infection with the Oroya strain were immune to the verruga strain. It is concluded therefore that Oroya fever and verruga peruviana are manifestations of infection with the same organism, the different clinical features depending on variations in the susceptibility of the host or the virulence of the parasite. The infection is transmitted by phlebotomus flies.

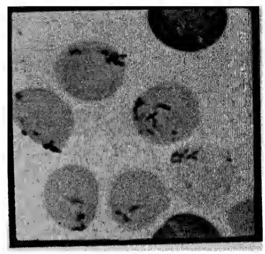


FIG. 192.—*Bartonella muris* in the blood of a rat nine days after splenectomy. Giemsa's stain. ×1200. From a preparation by Professor D. F. Cappell.

Bartonella muris.—Appearances in the red corpuscles of animals, similar to those of *Bartonella bacilliformis* have been described from time to time; certain of them have been termed *Anaplasma* (found in "gall sickness" of cattle in Africa) and *Grahamella* (in red cells of normal moles). Lauda found that they developed very constantly in the blood of rats a few days after splenectomy, and that there occurred also a severe anæmia, accompanied by fever and leucocytosis, death of the animal often following. The bodies which appeared within the red cells were called *Bartonella muris* (Fig. 192). McCluskie has verified this observation in mice after splenectomy. It is supposed that the animals harbour *Bartonella* in a latent state, and that splenectomy by reducing their resistance leads to an active infection. The view that the condition is an infection is supported by the fact that rats from South Italy did not show *Bartonella* after splenectomy, but when they were injected with blood or organ emulsions of susceptible Viennese rats and then subjected to splenectomy, they developed *Bartonella* anæmia. Cultures have not yet been obtained. The exact nature of these bodies has not been determined.

CHAPTER XXVIII

PATHOGENIC FUNGI

In medical bacteriology, besides the bacteria themselves, higher organisms belonging to the group of fungi (including moulds and yeasts) not infrequently claim attention. On the one hand, cultures may be contaminated with the spores of the omnipresent terrestrial forms growing in decaying material, and, on the other hand, certain fungi are known to be the causal agents in certain diseases. Before considering the latter, with which we are more intimately concerned, we shall first give a short account of the group of fungi as a whole and of some of the common saprophytic forms.

The majority of fungi consist of tubular branched filaments, termed *hyphæ,* each of which has a thin continuous wall within which are the protoplasmic and other contents. The whole body of the fungus thus composed of hyphæ is termed the *mycelium.* This may be loose and web-like in texture, as in the case of common moulds, or may assume the form of a compact skin or mass which is produced by the copious branching and close interweaving of the hyphæ, as in ordinary toadstools.

Phycomycetes.—In this lowly organised group of fungi, the hyphæ are usually continuous tubes devoid of any cross septa, excepting where reproductive organs or cells occur ; whereas in the more highly organised fungi the hyphæ are segmented by transverse walls.

Inasmuch as fungi are related to the algæ, which are mainly aquatic, those fungi that are most alga-like betray in their life-history signs of the aquatic mode of existence. Thus in a number of *Phycomycetes* the ends of certain hyphæ become shut off by a transverse wall. The terminal chamber becomes enlarged and its abundant protoplasm divides into a number of cells, which, by rupture of the outer wall, escape as naked ciliated *zoospores.* Each of these swims about in water (*e.g.* raindrops), eventually clothes itself with a thin cell-wall, and, emitting a hypha which grows and branches, developes into a new plant. The terminal organ within which these *asexual spores* arise is termed a *sporangium.* In other types of *Phycomycetes,* for instance *Mucor Mucedo* (Fig. **193**, A4), the spores arising in the same manner inside a sporangium, acquire a cell-wall before rupture of the sporangium wall : in this case the walled spores are adapted for dispersal through the air.

Some of the *Phycomycetes* can produce spores asexually in an entirely different manner, namely, externally by abstriction from

the end of a hypha. Such asexual spores externally cut off are termed *conidia*, and the special hypha bearing the conidia, if different in form from the vegetative hyphæ, is termed a *conidiophore*. Each conidium can emit one or more hyphæ and thus give rise to a new plant.

Other forms of asexual spores occurring in these simple fungi include *oidia*, in which a hypha undergoes cross septation into a number of short segments, each of which acts as an asexual spore. A hypha in this oidial condition has a resemblance to a greatly magnified row of bacteria ; indeed according to one theory bacteria represent merely oidial phases of "degraded" fungi.

Finally, as opposed to the thin-walled asexual spores so far mentioned, thick-walled asexual spores (often termed *chlamydospores*) occur in some of these simple fungi, and are endowed with greater powers of resistance to hostile external conditions and act as resting-spores

Phycomycetes also reproduce sexually. In the simplest case, as represented by *Mucor Mucedo*, the ends of two hyphæ come into contact and the terminal parts of the hyphæ are segmented off by a transverse wall The wall at the region of contact of the two hyphæ is dissolved, and the protoplasmic contents of the two terminal compartments fuse and produce around the resultant mass a thick wall. This thick-walled structure is capable of growing out to produce a new plant. As it is produced by the fusion of two structurally similar sexual cells it is termed a *zygospore* (Fig. 193 A1–3). Those *Phycomycetes* which have no marked structural distinction between male and female cells or organs, and whose sexually produced cells are therefore zygospores, are grouped together to form the class *Zygomycetes*. In some species of *Zygomycetes*, despite the similarity of the sexual organs, certain individuals (e.g. *Mucor Mucedo*) are physiologically unisexual, being either ♂ (male) or ♀ (female), and a zygospore results only when the hyphæ of the two such individuals fuse : in other species the individual is bisexual and can produce zygospores by the fusion of its ♂ and ♀ sexual organs.

In other *Phycomycetes* there is a very clear distinction between, on the one hand, the large female organ, which encloses one or more female cells, the ova or *oospheres*, and, on the other hand, the usually smaller but differently shaped male organ, which contains the equivalent of a number of male cells. The union of some of the protoplasm, including nuclear material, of the male organ with an oosphere results in the production of a fertilised egg-cell or *oospore*. Those *Phycomycetes* having this mode of sexual reproduction are grouped together to form the class *Oomycetes*.

Sexually produced cells, zygospore and oospore, germinate vegetatively to produce a new mycelium, or in a fructificative manner to produce a sporangium. The number of spores inside a sporangium of a phycomycete is often not only considerable but also variable in the same species. Thus if a plant of *Mucor Mucedo* be starved, the number of spores produced in each sporangium is reduced. Similarly in the *Phycomycetes* the number of conidia produced on a conidiophore is considerable and variable.

Ascomycetes and Basidiomycetes.—The more highly organised fungi differ from the *Phycomycetes* in that (1) they produce characteristic reproductive structures, *asci* or *basidia* ; (2) the hyphæ

are septate, with numerous cross partitions ; (3) the sexual process is reduced or modified and difficult to recognise. In any case, these fungi never, as an immediate result of a sexual fusion, produce a well-defined zygospore or oospore capable of developing into an independent vegetative fungus.

Two main divisions are recognisable among these fungi. In one series the sporangium has become " definite " in type, as it produces inside it a number of spores that is definite and constant to the species. The number of spores is usually eight, but a few species produce other multiples of two. This definite sporangium is termed an *ascus*, the spores are *ascospores*, and the group of fungi having asci are named *Ascomycetes*. In some of the *Ascomycetes* the asci are grouped together and form a kind of fruit-body (*ascocarp*), which, to give an example, is a closed spherical body in *Aspergillus* (*vide infra*). The production of asci may be preceded by a sexual fusion.

In the other division there is a characteristic reproductive structure, *basidium*, which usually bears four conidia or, in a few species, two or a multiple of two. This characterises the class *Basidiomycetes*, of which the common toadstools are examples.

The Smut- and Rust-fungi, *Ustilaginaceæ* and *Uredinaceæ*, are very simple *Basidiomycetes*, and among the salient features belonging to the members of this division is their capacity to produce thick-walled resting-spores, which in germination give rise to the basidium that buds off respectively indefinite and definite numbers of *basidiospores*.

In the *Basidiomycetes* the production of the basidium is a belated result of a sexual act in which the male and female nuclei do not fuse until long after the original protoplasmic fusion. In some species of the higher types an individual mycelium grown from one basidiospore will not give rise to the complete fungus with its fruit-body unless fusion takes place with another mycelium of similar origin : there are, then male and female individuals. In other species one basidiospore can give rise to the complete fungus with its fruit-body, probably because the mycelium can produce sexual nuclei.

Fungi Imperfecti.—The life-histories of some fungi placed in the groups already enumerated are incompletely known, yet certain characteristic stages are known, so that it is possible to refer these types more or less to their correct systematic position and class. For instance, the group *Chytridiinæ* includes a number of minute parasitic fungi, some of which are even devoid of a mycelium. Such forms may now be grouped in a separate division, designated *Archimycetes*.) But there still remain many kinds of fungi that are known only in their conidial stage. These imperfectly known fungi cannot be placed in their natural classes and have to be empirically grouped according to characters such as the arrangement and form of their conidiophores, structure and colour of their conidia. They are placed in the large unnatural group of *Fungi imperfecti*. Further, there are a few fungi known only in a mycelial condition. As a number of pathogenic fungi belong to the *Fungi imperfecti*, it may be pointed out that certain conditions must be fulfilled if a fungus is to complete its life-history. Many parasitic *Uredinaceæ* require two hosts. The completion of sexual reproduction may require certain environmental conditions. Very

commonly some degree of desiccation promotes sexual reproduction and the formation of fruit-bodies. In some cases the latter are not perfected in the absence of light. Finally, it is possible that some pathogenic *Fungi imperfecti* are male or female strains, and will not yield characteristic fruit-bodies until they are brought into contact with their opposites.

We now give examples of common non-pathogenic types.

Mucor Mucedo (and other species of Mucor).—This organism is assigned to the *Zygomycetes* (*vide supra*). It occurs on damp bread, horse dung, and other organic substrata. To the naked eye it appears as a white or smoky mould composed of fine filamentous usually non-septate hyphæ spreading over the substratum. Here and there arise erect hyphæ which in a saturated atmosphere may attain a length of several inches, but which are very much shorter in ordinary air. Each erect hypha ends in a spherical sporangium whose protoplasm is separated off from that of the supporting hypha by a transverse wall with a pronounced convexity toward the cavity of the sporangium, which forms the so-called *columella*. The protoplasm of the sporangium divides into many masses, each of which acquires a cell-wall and is then a spore. The spores escape by the rupture of the wall of the sporangium. (The needle-like bodies often seen outside the wall of the sporangium are crystals of calcium oxalate.) The less frequent sexual method of reproduction with the formation of the zygospore has already been described. The infrequency of the sexual mode of reproduction is due to the fact that the individual plants are sexually differentiated and might be termed male and female. Zygospore and asexual spore alike germinate to produce a new mycelium. In rich culture media or old cultures the mycelium may become septate. Cultivated under water some species (including *Chlamydomucor racemosus*) enter into an oidial condition (*vide supra*). See Fig. 193 A1–4.

Aspergillus Herbariorum.—This fungus is classified among the *Ascomycetes*. With other members of the same group, it is of frequent occurrence, especially on dead vegetable matter. It grows readily on culture media and, to the naked eye, consists of a mass of filaments which microscopically are seen to form a septate branching mycelium (Fig. 193 C1). Two forms of reproduction occur, the variety depending largely on the nutrition of the plant. The less common form is effected by means of structures known as ascocarps, which owe their formation to a sexual process. From a mycelial branch there arises a hypha which becomes specially coiled and traversely septate at its end. From the base of the lowest coil of the spiral two or three hyphæ grow up towards its apex, where one of these fuses with the coiled hypha and represents the male organ. The others by branching copiously produce a mass of closely woven hyphæ forming a closed wall to this structure, which is the ascocarp referred to. Within it numerous asci arise as the ultimate ramifications of branches given off by the central coiled hypha. Inside each ascus eight ascospores are produced. Ultimately all the structures lying within the ascocarp, save the spores, undergo disintegration, so that the mature ascocarp consists of a small hollow sphere within which lie the loose spores. These latter are ultimately freed by the decay of the wall of the ascocarp

and develop into new individuals. The commonest method of reproduction is by the formation of spores in the form of conidia, which are clearly of non-sexual origin. A filament grows out, and at its termination a rounded swelling is formed on which a series of little finger-like processes called *sterigmata* are placed. At the free end of each of these, rows of oval conidia are successively abstricted (Fig. 193 C2, 3). Each conidium, on becoming free, can give rise to a new individual, just as can an ascospore.

Penicillium.—This is a further representative of the *Ascomycetes*. It is the most common of all fungi met with in bacteriological work and comprises a large number of species, e.g. *P. expansum*, the common green mould found on decaying food, and *P. camemberti*, the green cheese mould. The mycelium is like that of the *Aspergillus*. Ascocarp formation takes place in a few species only, and the commonest mode of reproduction is by the conidia. A filament (the conidiophore) grows out, and at its end breaks up into a pencil of finger-like branches. On the point of each of these a peg-like sterigma is developed. On the end of this oval conidia are successively cut off; these break off and can give rise to new individuals (Fig. 193 D).

Saccharomycetaceæ or Yeasts (*Torula*).—These organisms have been subjected to much investigation in consequence of their economic importance in brewing and baking. They occur in nature chiefly in connection with fruits, such as the grape, which contain fermentable sugars. They consist of round or oval cells, 3 to 5 μ in longest diameter, and under ordinary conditions reproduce themselves by budding, in which process a portion of the cell protrudes, increases in size, and finally becomes separated from the parent cell so as to form a new individual (Fig. 193 E1). Under certain conditions of moisture and oxygen supply, endogenous sporulation occurs (Fig. 193 E2). As the spores produced are definite in number—two in some species and four or eight in others—the sporangium is an ascus and *Saccharomycetaceæ* may be regarded as ascomycetes. In certain species conjugation of two yeast cells and fusion of their nuclei take place: this process may immediately precede the production of an ascus. While in yeasts generally the oval cell represents the vegetative unit, in certain species elongated tube-like bodies may be formed which suggest an attempt at hypha formation. In some species the vegetative cells are so elongated and linked as to form a kind of simplified mycelium.

Oidium lactis.—This is a representative of the *Fungi imperfecti*. It is a common fungus in sour milk and sour bread, and can easily be cultivated on medium, where the colonies consist of short and fine septate filaments radiating from a centre. Here and there the hyphæ are divided, especially at the ends, into short oval or cylindrical segments, termed oidia, which act as spores. No other method of reproduction is known (Fig. 193 B).

Diseases produced by Fungi

Tinea and Favus.—In dealing with the common fungal infections of the skin, it is only possible here to give a short account of the methods employed in the investigation of the

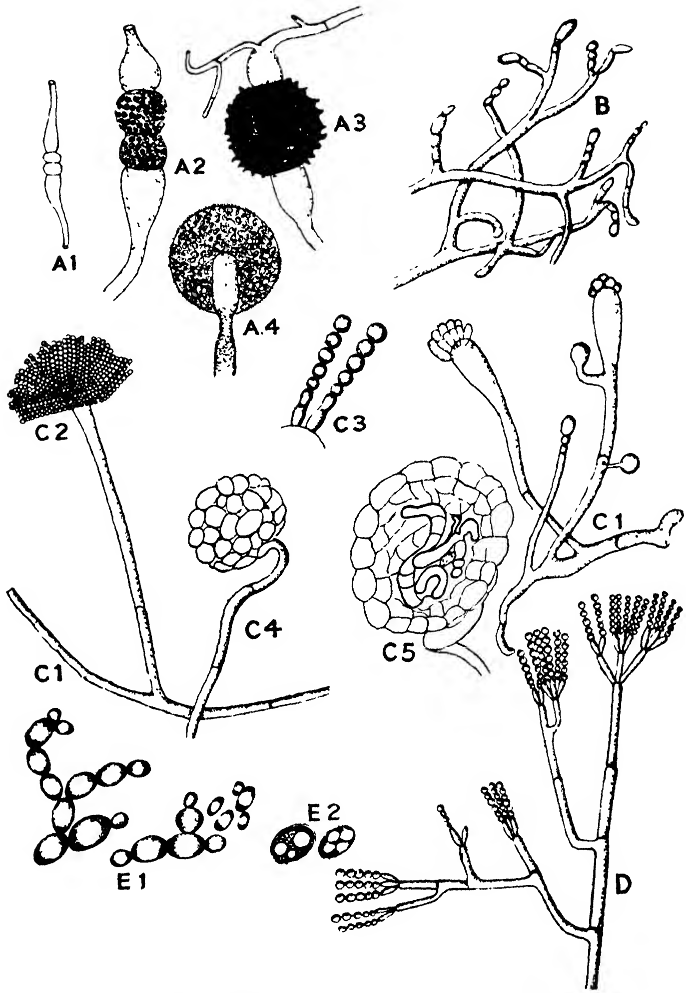


FIG. 193.—(After De Bary, Brefeld, and Hansen).

A. *Mucor Mucedo*; (1), (2) and (3) stages in formation of zygospore ×150; (4) sporangium containing spores ×150. B. *Oidium lactis* ×1000. C. *Aspergillus*; (1) mycelium ×100; (2) conidiophore with spores × 100; (3) two sterigmata with spores × about 600; (4) perithecium × 100; (5) perithecium with rudimentary asci × 200. D. *Penicillium*; branched conidiophore bearing spores × 400. E. *Saccharomyces cerevisiæ*; (1) budding cells × about 1000; (2) formation of endospores × about 1000.

more common types of fungi isolated. These organisms have been classified among the *Fungi imperfecti*.

Methods.—For ordinary purposes of diagnosis it is usual to place the epidermic scales or hairs in a solution of 7 grams of potash in 100 c.c. of water (Adamson) or in liq. potassæ (B.P.), to heat for a few seconds and to examine under a cover-glass. For permanent stained preparations Sabouraud recommends that the fat should first be removed by means of chloroform from the material, which is then placed in formic acid and heated for two or three minutes till the fluid boils. The acid is removed by washing in distilled water and the preparation stained for a minute with Sahli's blue, which has the following composition—distilled water, forty parts ; saturated aqueous solution of methylene-blue, twenty-four parts ; 5 per cent. solution of borax, sixteen parts. The preparation is then washed, dehydrated in absolute alcohol, cleared in xylol, and mounted in balsam.

The glucose and maltose media of Sabouraud (p. 76) constitute the best means of isolating skin fungi, as by these not only are the most characteristic growths obtained, but there is a certain degree of inhibition of the skin cocci. Where, as in tinea circinata, there is a vesicular or pustular lesion, the contents are squeezed out and transferred with a platinum wire to the medium. If there is a skin scurf, the squames may be scraped off on to a sterile slide from which tubes may be inoculated. When hairs are to be dealt with, these may be picked out on to a sterile slide, their roots cut off with a hot wire and planted in the medium. In certain hair affections, especially in animals, the parasite is specially abundant in the free part of the hair, so that portions of this, as well as the root, ought to be used. It is often advisable, especially in pustular conditions and in favus, to place the hair in absolute alcohol for two minutes, to allow to dry and then plant on the medium.

Microspora.—These are the small-spore ringworm parasites, and are responsible for a large proportion of the ringworms of the scalp occurring in children ; they only occasionally cause affections of the other parts of the body. In the initial lesion in the epidermis a fine mycelium, 1–5 μ in diameter, composed of rectangular elements, may be observed. This mycelium penetrates into the hairs where they emerge from their sheaths, and grows up and down in them. When an infected hair is examined, it is found to be encased by a mass of spores which have the characters of an irregular mosaic, the elements being frequently crushed together in polygonal forms and showing no tendency to an arrangement in rows. These spores are about 2 μ in diameter, but in potash preparations may appear larger—up to 5 μ. According to Sabouraud, the appearance on the hair results from intra-capillary mycelial threads breaking out at numerous points on the surface and there undergoing division

to form the spores. The mycelium can be demonstrated by mounting the hair in potash solution (*vide supra*) and disengaging the adherent and obscuring spores by gently rubbing the hair between the slide and the cover-glass. In culture these organisms may show hyphæ consisting of racquet-shaped cells ; spores are formed laterally and externally on the hyphæ. Large fusiform elements (" fuseaux ") divided by transverse septa, are also characteristic of this genus. The species most commonly present is the *Microsporum audouini* (Fig. 194), and a number of allied species have been isolated in the dog (*M. lanosum*), the cat (*M. felineum*), and the horse (*M. equinum*),

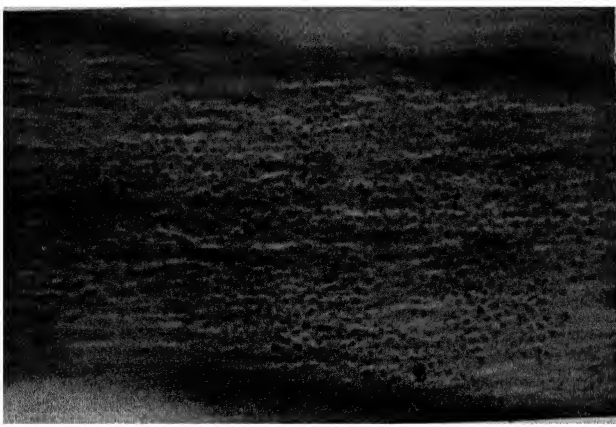


FIG. 194.[1]—Hair infected with *Microsporum audouini*. Photograph of unstained preparation. ×500.

and these are of importance from the frequent infection of man from such animal sources. Other species, e.g. *M. velveticum*, *M. umbonatum*, and *M. tardum*, presenting cultural differences, have been observed in man.

Trichophyta.—These fungi are associated with ringworm of the scalp, with the various manifestations found in the beard, and with the conditions occurring on the smooth parts of the body and in the nails. They are characterised by the fact that the mycelium, wherever observed—whether in epithelial squames, in pus, or within a hair—consists of chains of oval or

[1] For the photographs for Figs. 194, 201, 208, we are indebted to Dr. R. Cranston Low.

rectangular elements which have been generally spoken of as spores (Fig. 196). These in the largest forms are from 5 to 8 μ in diameter, but smaller forms approaching the size of the spores in *Microspora* also exist. There is thus not the same differentiation between mycelium and spores as seen in the *Microspora*, nor does the irregular mosaic appearance of the spores in the latter come into evidence. There is, however, the same primary affection of the superficial epithelium, and in hairy parts the same invasion of the hair where it emerges from its sheath. In cultures lateral spores are seen, either sessile or borne on a short sterigma.

FIG. 195.—*Microsporum audouini* on Sabouraud's maltose agar.

In certain species there is a tendency for the parasite to invade the follicle by growing down between the hair and its sheath for a considerable period before the hair itself is invaded—the so-called *Trichophyton ectothrix*. In the *T. endothrix* group the fungus continues to grow in the hair shaft after growth in the root sheath has stopped. A great number of *Trichophyta* presenting different cultural characteristics have been isolated. These are associated with differences in site of election and in method of spread in different parts of the body. There is evidence that certain varieties are more common in some countries than in others; for instance, in France *Trichophyton acuminatum* is the commonest, whereas in Scotland *Trichophyton crateriforme* (variety *flavum*) (Fig. 197, *a*) is the most frequent species in ringworm of the scalp, and *Trichophyton rosaceum* (Fig. 197, *b*) in ringworm of the beard. In France another coloured variety—*Trichophyton violaceum*—is of common occurrence. Similar organisms have been described

in the lower animals, such as the horse, calf, and dog, and the infection of man from such sources is relatively frequent.

The pathological lesions produced by the *Microspora* and *Trichophyta* are similar, though those of the latter are the more severe. In each case there is primarily a premature detachment of epithelial squames with subjacent inflammation in the corium, frequently followed by a slight hyperkeratosis, especially marked around and within the hair sheaths. Follicular pustules

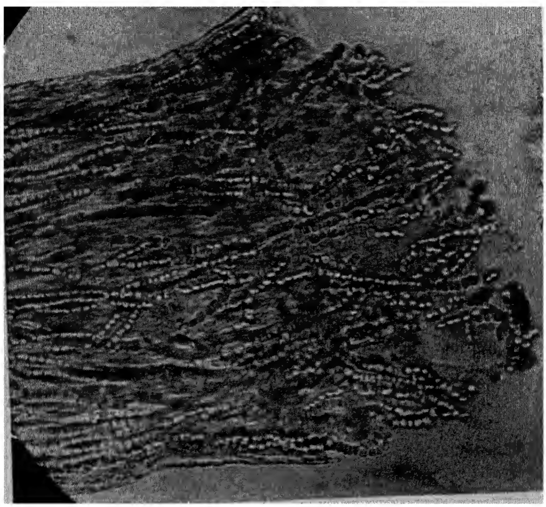


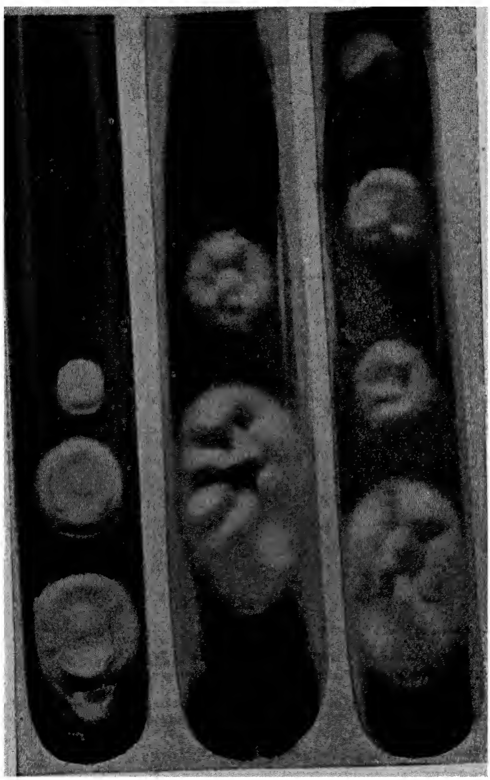
FIG. 196.—Hair infected with *Trichophyton*. Photograph of unstained preparation. ×500.

Note.—The sizes of the "spores" in Figs. 194 and 196 are not comparable, as in photographs of such thick preparations it is impossible to focus the outlines sharply.

are also common and in the most severe trichophytal cases a granulomatous condition (kerion) of the true skin, with relatively massive follicular suppuration, occurs. *Trichophyton* infection, especially when deep-seated, may give rise to a state of allergy (*vide infra*).

Achoria.—These organisms are responsible for the various clinical manifestations grouped under the name of favus, which affect both the hairy and smooth parts of the body. The characteristic of these is the development of round sulphur-yellow

discs (*scutula*), each with a depression in the middle, which in hairy parts often corresponds to the position of a hair follicle. These discs really consist of dense masses of fungal growth (Fig. 198). The initial change is a vigorous invasion of the epi-



a *b* *b*

FIG. 197.—*a*, *Trichophyton crateriforme*. *b*, *Trichophyton rosaceum*. Sabouraud's medium.

thelial squames, sometimes accompanied by an intra-epidermic, very often circumpilary, suppuration. As in the conditions previously described, the hair becomes invaded, the shaft being especially affected, but the hair infection is of subsidiary importance. The feature of the affection is the destruction of skin

structures (*e.g.* hair follicles), this leading, when recovery takes place, to the affected part assuming a cicatricial character. Sometimes a granulomatous affection of the skin is observed, which may be due to secondary infections. Preparations from the crusts (Fig. 198) show the presence of spores and mycelial threads, whose elements vary much in size and shape, but which are generally larger than those of the trichophyta. The affection of the hairs is severe, and the track of the mycelium is often marked by the presence of comparatively large air channels

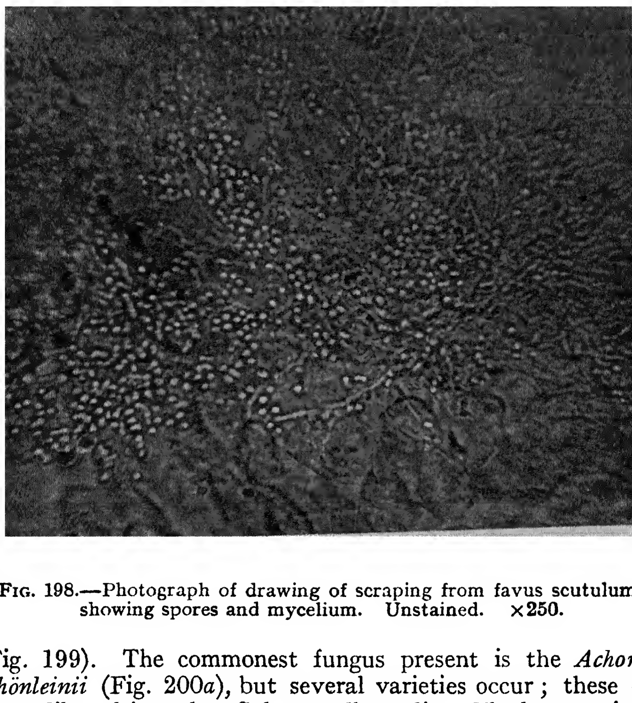


FIG. 198.—Photograph of drawing of scraping from favus scutulum, showing spores and mycelium. Unstained. ×250.

(Fig. 199). The commonest fungus present is the *Achorion schönleinii* (Fig. 200*a*), but several varieties occur; these can be readily cultivated on Sabouraud's media. The lower animals (mouse, dog, cat, fowl (Fig. 200*c*)) are often affected. The type commonly found in the mouse is *Achorion quinckeanum*, and it may also occur in man.

Epidermophyta.—Superficial inflammatory lesions of the skin due to infection with fungi of the genus *Epidermophyton* have become increasingly common during the past twenty years. There are a number of different species, the commonest being

E. cruris and *E. rubrum*. These fungi occur as long undulating branching hyphæ in the most superficial layers of the epidermis (stratum corneum). The hyphæ may be composed of short oblong segments 4 to 12 μ long by 4 to 5 μ broad, giving the whole thread a somewhat beaded appearance (Fig. 201), or segmentation may only occur at infrequent intervals. The fungus is confined to the epidermis, and does not invade either the strong or lanugo hairs. It is impossible to distinguish *Epidermophyton* from *Microsporum* or *Trichophyton* in preparations of epidermal scales. Cultures closely resemble those of many of the species of *Trichophyton*, but the microscopic elements differ in the two genera (*vide infra*).

As met with clinically, *Epidermophyton* has a predilection

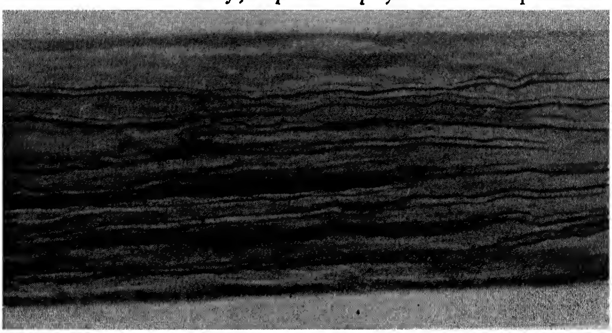


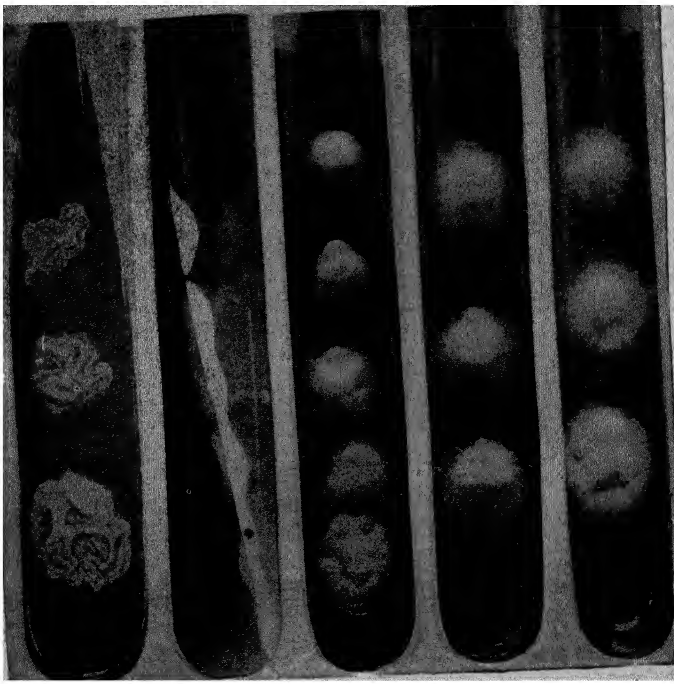
FIG. 199.—Favus hair showing air channels left by mycelium. ×300.

for the body folds, and the lesions which it produces almost invariably commence in this situation. The most common sites for epidermophytosis are the groin, as in the so-called Dhobie itch, and the interdigital spaces of the feet. Thence the infection may spread to involve adjacent areas or give rise to secondary lesions at a distance. Infection by this organism may produce supersensitiveness involving the entire skin surface, and secondary allergic eruptions, analagous to those met with in deep-seated *Trichophyton* infections, may be produced (*vide infra*). *Epidermophyton* infection frequently occurs in epidemic form in institutions. There is considerable evidence to show that infection takes place through the medium of some inanimate object such as clothing, rather than by direct contact.

Endodermophyton.—This includes only a few species (e.g. *E. concentricum*) occurring in tropical infections of the skin, such as

the so-called Tokelau ringworm first described by Manson. They attack the stratum corneum, producing lesions with " watered-silk " appearances.

Cultural characters of the skin fungi.—It is unnecessary to describe in detail the characters presented in cultures by the



a *b* *c* *c* *c*

Fig. 200.—*a*, Photograph of drawing of *Achorion schönleinii* on Sabouraud's maltose agar. *b*, Side view to show elevation of growth. *c*, Photographs of cultures of *Achorion quinckeanum* (The central culture of *c* was isolated from a cat, and the two others from a man infected from it.)

various groups of parasitic skin fungi, and we need only mention certain commonly occurring characters. In all there is a free production of a septate mycelium, and usually, by a lateral budding from the hyphæ or by the breaking up of the protoplasm of the thread, there is the formation of bodies resembling

those described as spores which occur in affected tissues. This spore formation often shows a tendency to occur specially at the termination of filaments. Sometimes in the course of a filament an element enlarges and from it new mycelia sprout, the whole resembling chlamydospore formation. Sometimes, especially in the *Microspora* and *Achoria*, large fusiform elements ("fuseaux") divided by transverse septa are observed. Curious spiral elements whose significance is unknown are also fre-

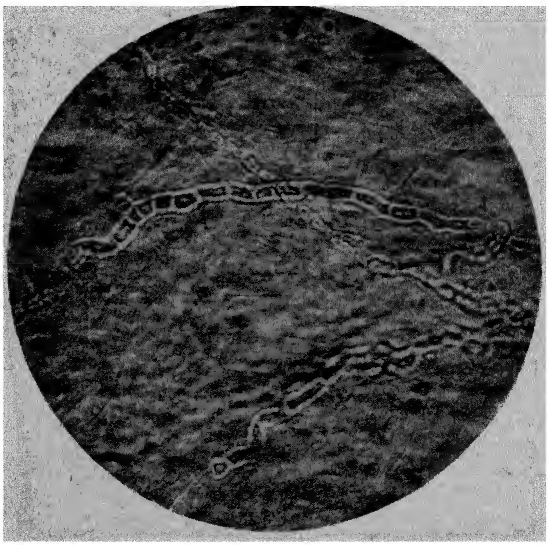


FIG. 201.—*Epidermophyton rubrum.* Unstained preparation from a case of epidermophytosis of foot. × approx. 1000. Photograph of a preparation by Dr. G. H. Percival.

quently seen. Among the *Epidermophyta* no lateral conidia are observed, and there are no sporing hyphæ; on the other hand numerous spindle-shaped elements are characteristic of this genus.

For a full description of the naked-eye characters of the various ringworm and favus fungi, the reader must be referred to such works as those of Sabouraud and of Castellani. The characters vary very much with the medium employed, and hence in any comparative study it is of great importance that the same medium should be used; and it is even necessary

that a large bulk of a medium should be made up at once so as to be available for an extended study.

On Sabouraud's media most of the fungi at the commencement of their growth appear as white fluffy or felted button-like colonies on the surface, and as growth proceeds differentiating characters emerge. Thus the organism may tend to spread in a fairly thin layer over the medium and sometimes there is an appearance of successive concentric rings of growth; on the other hand, the colony may be heaped up in the centre as a projecting knob, or there may be a central depression round which the heaping up occurs. Sometimes there are ridges or folds radiating from the centre of the colony, presenting a geometrical arrangement or having an irregularly convoluted appearance. The surface may have a woolly character or may give the impression of being covered with fine powder. Sometimes the surface formation is moist and slimy-looking. These appearances are exemplified in Figs. 195, 197, 200. When colour is produced it develops with age. An important point is the occurrence of variation. Thus a subculture frequently presents characters different from those of the parent growth, or on a coloured colony colourless points may appear which may maintain the non-pigmented character when subcultured. Variants may also develop as sectors of the parent colonies. On media presenting large surfaces the colonies assume a correspondingly large size, and growth usually goes on until the medium is exhausted.

Generalised Lesions.—Within recent years it has come to be recognised that the conditions just described are not always merely local lesions, as was formerly supposed, but that occasionally in both types of ringworm (especially in deep-seated lesions due to *Trichophyta*) and in favus, generalised skin eruptions occur. These are known as trichophytides, microsporides, and favides respectively. The eruptions are of various kinds—papular, vesicular, pustular, etc., and their appearance is accompanied by polymorphonuclear leucocytosis, fever, etc.; they are manifestly hæmatogenous in origin. In a certain number of cases the parasite of the original infection has been found in the secondary lesions, and in a few instances has actually been cultivated from the blood. The view has, accordingly, gained acceptance that the fungi in question may occasionally become disseminated by the blood stream and arrested in the blood vessels of the cutis, where their toxins act on an allergic skin. In many instances, however, there is no evidence of the presence of the parasite, and the rash is of such a nature, for example

scarlatiniform, that it is hardly possible it has been produced in this way. It is, apparently, a reaction of supersensitiveness, due to the products of the parasite, and it is to be noted that allergy of the skin has been demonstrated in such cases. Bloch, for example, produced scarlatiniform and lichenoid eruptions by intravenous injection of trichophytin (*vide infra*) in cases of kerion.

Immunity and Supersensitiveness.—A large amount of work has been done on these subjects in recent times, but many of the results are conflicting; the following is a short summary of what seems to be established. General reactive phenomena indicated by supersensitiveness and the presence of antibodies in the blood are met with in the fungus infections, but only when there is considerable involvement of the connective tissues, *e.g.* in deep-seated ringworm, sporotrichosis, hemisporosis (*vide infra*), etc. The reactions are analogous to those which are recognised as occurring in granulomatous infections, *e.g.* tubercle, and are not attended by an immunity to the invading parasite. Evidence of supersensitiveness or allergy is obtained by injection of extracts of the parasite. For example, in deep-seated ringworm especially such an extract (trichophytin) produces a local, a focal, and a general reaction, the last being attended by pyrexia, etc. (cf. tuberculosis, p. 432). The presence of antibodies such as agglutinins, complement-fixing bodies, etc., has been observed in infections with much tissue change, such as those mentioned above. It is to be noted, however, that such reactions are not strictly specific, as they are given, though often in less degree, with fungi other than the invading organism, the results indicating a certain degree of common antigenic structure of the fungi.[1]

Piedraia hortai (*Trichosporum*).—This organism produces "piedra" a nodular condition of the hair. The nodules are made up of closely bound mycelium along with a greenish-yellow viscous product. Asci, usually with eight spores, are formed. The fungus can be cultivated on Sabouraud's medium and develops in mycelial formation but without asci. Unlike the ringworm fungi this organism grows only on the hair surface. The ascospores readily escape in the presence of water.

Thrush (German, *Soor*; French, *Muguet*).—This condition, which is most common in children, chiefly affects the tongue and fauces, and may extend into the œsophagus. It is characterised by white patches largely composed of fungal growth,

[1] For details, the reader may be referred to *Anaphylaxis and Sensitisation*, by R. Cranston Low, Edinburgh, 1924.

which cause catarrh of the subjacent epithelium and slight erythema. A similar condition may occur in the vagina, and a few cases of generalised affection with abscesses or tubercle-like lesions in the solid organs, *e.g.* the lungs, have been recorded. The organism has been called *Oidium* (or *Monilia*) *albicans*. Both in the tissues and in cultures the chief elements are double-contoured, septate mycelial threads—the elements being of varying sizes—and round or oval yeast-like forms (Fig. 202) developed by budding from the mycelium or from chlamydospores produced at the ends of certain filaments. These yeast forms may be 5–6 μ in their long diameter. The fungus grows readily on artificial media, especially those containing beer-wort (p. 76), and while some varieties liquefy gelatin, others do not. In the case of the latter, the superficial colonies on gelatin are granular with peripheral feathery extensions, while the deep colonies are rounder and more circumscribed. The colour is white or slightly red, and the cultures have a sourish alcoholic smell due to the production of aldehyde, alcohol, and acetic acid; glucose, lævulose, and maltose are slowly fermented, but the fermentation reactions vary in different types. On ordinary media, mycelium and yeast-like cells are seen, the former being especially marked in deep colonies. Formation of chlamydospores may also occur.

Fig. 202.—*Oidium albicans*. Film from culture stained fuchsin. ×500.

Reference is made above to the classification of the thrush fungus in the genus *Monilia* of the *Saccharomycetaceæ*, and it may be noted here that this designation has been applied to organisms which show both yeast-like forms and also hyphæ without ascospore development; these organisms are also active carbohydrate fermenters and produce both acid and gas in their fermentation processes.

Organisms of this type have been described in other conditions e.g. *M. psilosis* in sprue, *M. tropicalis* in bronchial infections ("broncho-moniliasis"), in circumscribed dermatitis, suppurative paronychia etc. It is doubtful whether *M. psilosis* is the etiological agent of sprue.

Aspergillosis.—In 1856, Virchow recorded several cases of affection of the lungs by aspergilli, and a number of similar cases have since been described; usually there has existed some other disease in the body, and frequently the lung has also been the site of tuberculosis. The appearances presented are those of small grey nodules composed of necrotic material and leucocytes, which break down to form cavities associated with areas of broncho-pneumonia, and frequently also with fairly widespread odourless necrosis of the lung. Masses of fructifying mycelia are present in the cavities and extend into surrounding bronchioles and air cells. The condition has usually been discovered *post mortem*, but in certain cases the fungus has been observed in the sputum during life, and it is probable that a lung condition of this kind can be recovered from. A similar affection occurs in birds. It is probable that infection arises from inhalation. The variety of organism most frequently found is the *Aspergillus fumigatus* (cf. p. 861), which on artificial media gives a greenish-blue colour. Its optimum temperature is that of the body.

Infections with aspergilli also occur in the external ear in the form of chronic pustular conditions, and colonies of aspergilli are also from time to time met with on abrasions of the cornea.

Certain varieties of fungi associated with mycetoma (*vide* p. 480) have been classified as aspergilli. An aspergillus (*A. pictor*) occurs also in a skin disease observed in Central America, known as Pinta.

Sporotrichosis.—In 1898, Schenk, in America, described a case of chronic subcutaneous abscesses associated with a fungus belonging to the *Sporotricha* (classified among the *Fungi imperfecti*), and during recent years the organism has been isolated from granulomatous conditions occurring in various parts of the world. Most of the cases have been characterised by somewhat heteromorphic and indolent granulomatous lesions in the skin, resembling those of tuberculosis and syphilis. The initial lesion is at the site of some slight abrasion, and it is followed by a succession of granulomata, usually small, whose distribution indicates a lymphatic spread. There is little tendency to spontaneous cure. Apart from the skin, cases have been recorded of lesions in the pharynx, larynx, muscle, bone, and synovial membrane; and both in man and in animals (dogs, rats) generalised infections of the serous cavities and solid organs have been observed. The lesions are of a diffuse granulomatous character, and at first consist chiefly of young connective-tissue elements with plasma cells and lymphocytes.

Later, diffuse degeneration and necrosis occur and also leucocytic emigration with the formation of abscesses, at first of microscopic size. When the skin is involved, ulceration results. In certain cases abscess formation is more marked. No generalised skin eruptions, such as occur in tinea and in favus, have been observed in sporotrichosis. The serum of patients suffering from the disease has been found to contain antibodies ; but the reactions are not specific, as they are given also with other fungi.

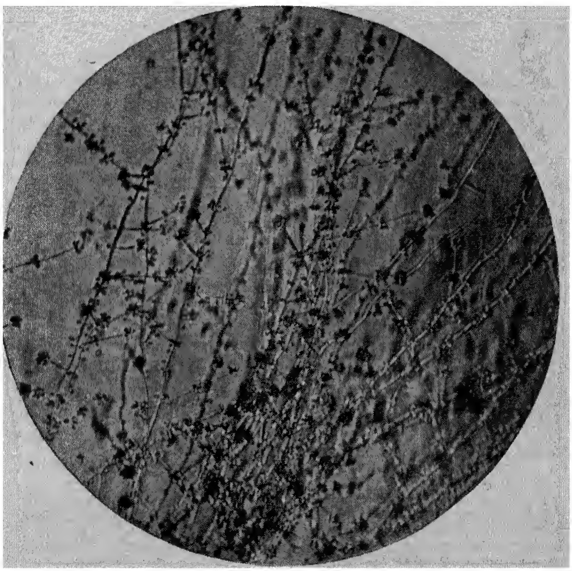


FIG. 203.—Edge of living colony of *Sporotrichum beurmanni* on agar hanging-drop, five days at 22° C. ×200.

Direct examination of the pus may reveal the presence of oval, highly refractile spores, 3–4 μ long and 1·6–3 μ broad, and these may be demonstrated both free and in the granulomatous cells, in films and sections stained by ordinary aniline dyes ; they are Gram-positive. Mycelial formation does not occur in the tissues, except occasionally in the most superficial parts of an ulcerating lesion. If a drop of pus be placed on the glass of an agar tube just above the condensation water, the development of mycelium from the spores may be directly observed with the microscope. The organism, which is generally known as

the *Sporotrichum beurmanni*, grows readily on any ordinary medium (gelatin, agar, potato), but is best studied on Sabouraud's medium. Two sets of media should be inoculated —one incubated at 37° C. and the other at room temperature. On the latter, after about forty-eight hours, somewhat fluffy, snowflake-like, white points appear which gradually become brown, and when growing in mass present a heaped-up convoluted growth. The morphology of the organism is best studied in hanging-drop preparations made with agar. From a spore a mycelial thread about 1 μ in thickness, irregularly septate, and often containing fine granules, develops. Lateral branches arise and fresh spore-formation is soon observed. These usually develop in whorls round a filament (Fig. 203), but sometimes the process occurs all along a filament. Sometimes, in the course of a filament large circular elements, 5–6 μ in diameter, resembling the zygospores of *Mucor* are seen, and these sometimes contain groups of spore-like bodies. The free growth of the organism depends on conditions of moisture and temperature, and where these are unfavourable, instead of mycelial formation being observed the spores may enlarge to three or four times their ordinary size and then give off circles of fresh spores. The appearances are frequently like those of a yeast (Fig. 204). Under a low power of the microscope, mycelial colonies have a stellate appearance with a very freely spiked edge. The organism manifests considerable vitality under saprophytic conditions, and is said to have a fairly widespread distribution. Cultures are pathogenic when injected subcutaneously in mice, rats, dogs, etc., granulomatous lesions identical with those of the natural disease being produced.

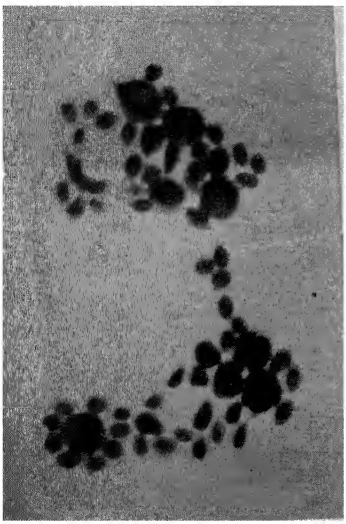


FIG. 204.—Film from agar culture of *Sporotrichum beurmanni* grown at 37° C. for ten days. Gram's stain. ×1025.

In the past sporotrichosis in man has probably often been confused with the manifestations of syphilis, as the condition readily yields to the administration of potassium iodide. In horses, certain cases presenting the characters of epizootic lymphangitis have been found to be associated with an organism indistinguishable from the *Sporotrichum beurmanni*.

Hemisporosis.—This is a fungus infection first described by Gougerot and Caraven who obtained from the lesions pure cultures of a parasite which was identified as the *Hemispora stellata* (Vuillemin). This group of organisms is assigned to the *Fungi imperfecti*. Since then a considerable number of cases has been reported, especially from Italy. Like sporotrichosis, it is characterised by the formation of granulomatous growths which may resemble syphilitic or tuberculous lesions, and which may occasionally undergo suppurative softening. There may be a single lesion, or there may be multiple lesions, *e.g.* nodules in the skin; occasionally the bones are affected. Gougerot and Caraven found that the serum of the patient gave agglutination and complement-fixation reactions with the parasite. These, however, are not strictly specific, as the reactions may be obtained with other fungi. They found that the organism had a similar pathogenic action in the rabbit.

The name *Hemispora stellata* was applied to the parasite owing to the star-like arrangement of the conidiophores on the surface of the mycelial growth, as seen under a low magnification. It is a fairly common saprophyte in nature, occurring in the dust of rooms, etc. On culture media it forms an irregular whitish layer of indefinite extent. Certain of the mycelial threads become somewhat swollen at their free extremity, forming "protoconidia," and these become segmented into 3–8 subspherical "deuteroconidia," measuring $2{\cdot}5\ \mu \times 3{\cdot}5\ \mu$, which constitute spores. It may be difficult to detect the organism by microscopic examination of the tissues, while cultures may be readily obtained on various media.

Rhinosporidium sieberi.—This organism has been found in a polypoid condition of the nose occurring in India and certain other parts of the world. The conjunctiva, lacrymal sac, uvula, ear and penis have also been reported as sites of the infection. The organism may now be classified among the *Archimycetes* (*vide* p. 860). The elementary structure is a spore-like body which in the mature state has a chitinous envelope, vacuolated protoplasm and vesicular nucleus. These bodies may be about 6–8 μ in diameter. By progressive enlargement, subdivision of the nucleus and cytoplasm, and by the formation of a cellulose capsule a structure resembling a sporangium is developed containing large numbers of new spores

(Fig. 205). This body may reach 0·25–3 mm. in diameter. The capsule has a single "pore" through which the spores are discharged; they then spread in the lymphatic spaces.

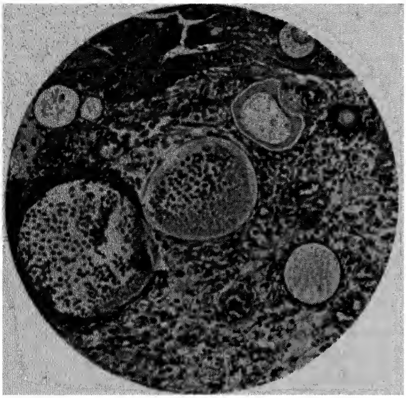


FIG. 205.—*Rhinosporidium* in section of nasal polypus. Stained iron hæmatoxyln and eosin. ×100.

Blastomycosis. — In pathological literature there are recorded a very large number of usually isolated cases presenting the characters of granulomata or of chronic suppurations, in connection with which the presence of yeast-like organisms has been observed, and from which cultures of these have been obtained. In the tissues the organisms usually appear as double-contoured cells which multiply by budding or by a process resembling endogenous sporulation. Two genera have been recognised in relation to these conditions: *Cryptococcus*, which is a typical yeast-like organism without any appearance of hyphæ and mycelium and without ascospores; and *Coccidioides* in which mycelium is formed and elements corresponding to ascospores are observed. *Cryptococcus gilchristi* is a species described in blastomycotic dermatitis (*vide infra*). *Coccidioides immitis* is the organism described in conditions of generalised blastomycosis—(*vide*

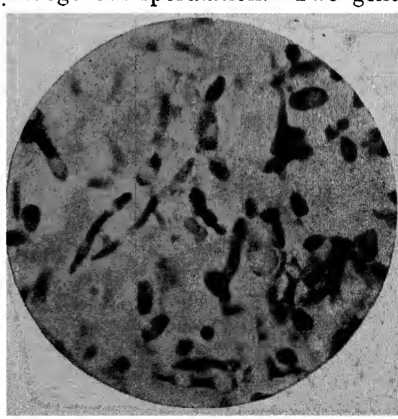


FIG. 206.—Growth of yeast-like organism in kidney of rabbit infected from human case (see text). ×1000.

infra). (The term "blastomyces," which may be taken as synonymous with yeast, finds no place nor has it any specific significance in modern descriptive mycology, for in vastly differing species yeast-like elements occur representing stages in development.) From their tendency to produce mycelia, certain of these organisms resemble the forms often described as *Oidium*, and the genus *Monilia* (*vide supra*) has also been grouped with *Cryptococcus* and *Coccidioides*.

It has been considered that *Coccidioides* is closely related to the organism designated *Rhinosporidium* (*vide supra*). Thus the spherical bodies seen in the tissues may reach 80 μ in diameter and may possess a thick and rough capsule. When mature this cell may be filled with small spore-like elements which ultimately escape into the surrounding tissue. The genus is, however, ill-defined and is generally classified among the *Saccharomycetaceæ*.

While organisms of this group have been isolated from many conditions, in which there is no evidence that they play an etiological rôle, there is no doubt that they can multiply and cause pathological changes in the animal body. An example of this is seen in Fig. 206, taken from the kidney of a rabbit which was inoculated subcutaneously with an organism isolated from the sputum of a human case of obscure granuloma of the lung, associated with a suppurative condition in the kidney and the presence of similar organisms in the urine. In this case the appearances of the organisms in the tissues corresponded to those seen in cultures.

Blastomycotic dermatitis has been widely studied in America, and especially in Chicago. The disease may arise in any part of the skin and frequently follows a slight wound. The development of a sluggish papule, becoming pustular and ulcerative, is followed by a slowly extending granular and papillomatous appearance, with irregularly distributed pustule formation, and surrounded by a reddened areola containing numerous miliary abscesses. Areas of this kind, several inches in diameter, may slowly develop. These may heal at one margin and extend widely at another. The process may go on for years, and various, it may be distant, parts of the skin may become successively affected. In the great majority of cases no general disturbance occurs. Microscopically, in the fully advanced stage, the picture is that of an irregular epithelial proliferation and hyperkeratosis with superficial papillomatous excrescences, and more deeply of a similar irregular and free epithelial proliferation taking place in a granulomatous condition of the cutis. Special features are the development of minute pustules, partly intra-

epithelial, partly in the corium, and the formation of giant cells. In the pus, organisms presently to be described are found. The disease is of chronic nature and is restricted to the skin.

Generalised Blastomycosis.—This condition was described by Wernicke in South America and by Rixford and Gilchrist in California. In the first described cases attention was directed to the occurrence of suppurative conditions in the lungs. A skin lesion also occurs, characterised by subcutaneous abscesses or granulomata leading to ulceration with epithelial hyperplasia. This may be the primary manifestation of the disease, but the internal organs become affected with chronic suppurative processes or granulomata, and death occurs. Cases belonging to the same class are also recorded where subcutaneous nodules, consisting of myxomatous connective tissue, have been observed, associated with the occurrence of suppurations in the internal organs. The cases of generalised infection were at first attributed to protozoa. The direct observation, under the microscope, of the growth of a mycelium from the protozoon-like body showed the fungal nature of the organism and led to its being designated *Oidium coccidioides* (now named *Coccidioides immitis*).

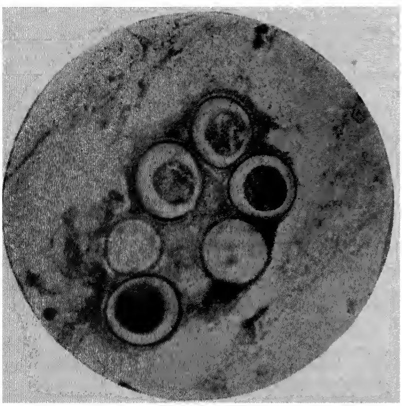


Fig. 207.—Double-contoured bodies in tissues from one of Rixford and Gilchrist's cases of Blastomycosis. ×500.

Examination.—In blastomycotic dermatitis the organisms (*Cryptococci*) are present chiefly in the abscesses in the corium, and can be demonstrated by mounting the pus in 30 per cent. caustic potash solution. They are spherical in form, 8 to 10 μ in diameter, and appear singly, in pairs, or less frequently in larger groups (Fig. 207). There is a central protoplasm without distinct nucleus, separated by a delicate membrane from a surrounding clear space, and the whole is enclosed in a highly refractile, double-contoured capsule. Budding is frequently seen. The organisms stain with hæmatoxylin and with basic

dyes and are Gram-positive, the reaction of the capsule being variable.

The facility with which the fungi have been cultivated varies in different cases, but growth can usually be readily obtained at room temperature or at 37° C. on ordinary media, but preferably on Sabouraud's maltose medium, especially when this is made slightly acid. Growth appears in from two to seven days, and the characteristics vary. In some cases moist, paste-like colonies develop, in others the surface appears crumpled, and sometimes it is dry and powdery. These differences are associated with differences in the degree of mycelial formation, in the extent of the ingrowth of the organism into the medium, and in the presence or absence of aerial conidia. The effects of the different varieties differ. Glucose and maltose are usually fermented; gelatin is ordinarily not liquefied; and indole formation is uncommon. In cultures the budding seen in the tissues is also observed, and there is a varying amount of formation of segmented and branching hyphæ, this in certain cases being particularly well marked and giving rise to a definite mycelium. Somewhat slender aerial hyphæ sometimes occur which may form lateral spherical conidia, and sometimes terminal bodies resembling ascospores. The elements in cultures resembling those seen in the tissues frequently also possess a double-contoured capsule.

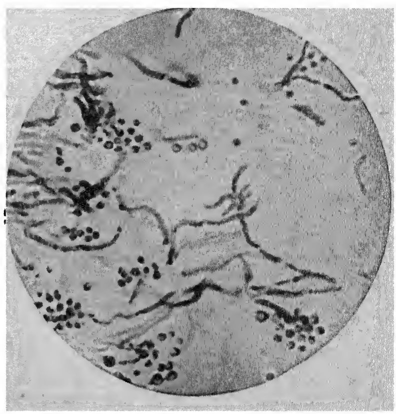


FIG. 208.—*Microsporum furfur*; scraping from skin. Gram's stain. ×200.

These organisms may be pathogenic for animals. Abscesses follow subcutaneous inoculation in guinea-pigs, rabbits, and mice, and death may result. Intravenous injection may result in a fatal pulmonary infection; intraperitoneal infection is often without result.

Cryptococcus and *Monilia* (pp. 875, 880) types are not infrequently observed in cases of circumscribed dermatitis and in paronychia. *Cryptococcus linguæ pilosæ* has been described in a "mycosis" of

the tongue known as "black tongue." A species of *Cryptococcus* sometimes classified as *Torula* (*T. histolytica*) has been described in meningitis.

Microsporum (or Malassezia) Furfur

This is the organism associated with *Pityriasis versicolor.* The condition, which is very widespread all over the world, occurring often in phthisical patients, is not looked upon as a disease of the skin, but is due to the saprophytic growth of the fungus on the skin surface. The organism can be demonstrated in scrapings from the lesion, either examined in potash solution or in films stained by, for example, Gram's method. The organism consists of an irregularly contoured crumpled mycelium in segments 7–13 μ long and 3–4 μ broad. Associated with this, there are irregular groups of double-contoured spore-like bodies 4–7 μ in diameter (Fig. 208). Nothing further is known regarding the organism, as most attempts at cultivation have had a negative result, and even where cultures are said to have been obtained it has been impossible to secure continued growth.

CHAPTER XXIX

THE BACTERIOLOGY OF AIR, SOIL, WATER, SEWAGE, AND MILK; ANTISEPTICS; CHEMOTHERAPEUTIC AGENTS

As this work deals essentially with bacteriology in relation to pathology and clinical medicine and surgery, its scope does not include a full account of the applications of the science to practical sanitation. It is convenient, however, to give an outline of some of the methods employed in sanitary work and to indicate the chief results obtained.

Air

Very little information of value can be obtained from the examination of the air, but the following are the chief methods used, along with the results obtained.

Methods of Examination.—The methods employed vary with the objects in view. If it be sought to compare the relative richness of different atmospheres in organisms, and if the atmospheres in question be fairly quiescent, then it is sufficient to expose agar plates for definite times in the rooms to be examined. In each case one plate is incubated at 37° C. and one at about 22° C. Bacteria, or the particles of dust carrying them, fall on the plates, and from the number of colonies which develop a comparative estimate of the richness of the air in bacteria can be obtained. Petri stated that in five minutes the bacteria present in 10 litres of air are deposited on 100 square centimetres of a plate.

More complete results are available when some method is employed by which the bacteria in a given quantity of air are examined. Thus such a quantity of air may be bubbled by an aspirator through sterile water, and measured amounts of the fluid may be plated in nutrient agar. Of the more formal apparatus which have been used the following may be described:

Petri's Sand-Filter Method.—A glass tube open at both ends, and about 3½ inches long and half an inch wide, is taken, and in its centre is placed a transverse diaphragm of very fine iron gauze; on each side of this is placed some fine quartz sand which has been burned, well washed, and dried to remove all impurities, and this is kept in position by cotton plugs. The whole is sterilised by dry heat. One plug is removed, and a sterile rubber bung

inserted, through which a tube passes to an exhausting apparatus. The tube is then clamped in an upright position in the atmosphere to be examined, with the remaining plug uppermost. The latter is removed and the air sucked through. Difficulty may be experienced from the resistance of the sand if quick filtration be attempted. The best means to adopt is to use an air-pump—the amount of air drawn per stroke of which is accurately known—and to have a manometer (as in Fig. 19) interposed between the tube and the pump. Between each two strokes of the air-pump the mercury is allowed to return to zero. After the required amount of air has passed, the sand above the diaphragm is removed ; plate cultures are then made from it, and when growth has occurred the colonies are enumerated ; the sand below the diaphragm is similarly treated, and acts as a control.

When it is necessary to examine air for particular organisms, special methods must often be adopted. Thus, in the case of the suspected presence of tubercle bacilli, a given quantity of air is drawn through a small quantity of broth, which is then injected into a guinea-pig.

Comparatively little information bearing on the hygienic condition of the air is obtainable by the mere enumeration of the living organisms present, for under certain conditions the number may be increased by the presence of many bacteria of a purely non-pathogenic character. The organisms found in the air belong to two groups—firstly, a great variety of bacteria, *e.g.* sporing bacteria of the *B. subtilis* group (*q.v.*), staphylococci of the *albus* type, chromogenic cocci, such as *Sarcina lutea* ; secondly, yeasts and the spores of moulds. With regard to moulds, the organisms consist of felted masses of hyphæ, from which are thrust into the air special filaments, and in connection with these the spores are formed. By currents of air these latter can easily be detached, and may float about in a free condition. With the bacteria, on the other hand, the case is different. These organisms are only present in the air when detached in dust from some dry substrate, or when introduced into the atmosphere from the exhalations of man and animals. The entrance of bacteria into the air, therefore, is associated with conditions which favour the distribution of dust and the presence of secretion droplets from the respiratory passages, etc. The presence of dust, in particular, would add a large number of bacteria to the atmosphere, and this is the case with the air in many industrial conditions, where the bacteria, though numerous, may be quite innocuous. On the other hand, there is no doubt that pathogenic organisms can be disseminated by means of the air. The possibility of this has been shown experimentally by contaminating the mouth with a culture of *B. prodigiosus*, which is easily recognised by its

brilliantly coloured colonies on culture medium, and then studying its subsequent distribution. The actions of coughing, sneezing, speaking, and even of deep breathing, distribute, often to a considerable distance, minute droplets of secretions from the mouth, throat, and nose, and these may float in the air for a considerable time. Even five hours after an atmosphere has been thus infected, evidence may be found of bacteria still floating free, though the majority have settled by this time. Examples of diseases in which infection can take place in this way are diphtheria, influenza, pneumonia, plague of the pneumonic type, and phthisis. In the case of phthisis, the deposition of tubercle bacilli has been demonstrated on cover-glasses held before the mouths of patients while talking, and animals made to breathe directly in front of such patients have become infected with tuberculosis.

With regard to infection by dust, a most important factor is whether or not the infecting agent can preserve its vitality in a dry condition. In the case of a sporing organism such as the anthrax bacillus, vitality is preserved for long periods of time, and considerable resistance to drying is also possessed by the tubercle bacillus. The aerial transmission of anthrax spores by dust and filaments from infected wool, *e.g.* in wool factories, has been well recognised (*vide* p. 496), and it is also well known that tubercle bacilli may be carried by dust. But apart from such cases there is little doubt that air infection is usually associated with the transport of secretion droplets, and is thus confined to a limited area around a sick person.

Soil

The investigation of the bacteria which may be found in the soil is undertaken from various points of view. Information may be desired as to the change its composition undergoes by a bacterial action, the result of which may be an increase in fertility and thus in economic value. Under this head may be grouped inquiries relating to the bacteria which convert ammonia and its salts into nitrates and nitrites, and to the organisms concerned in the fixation of the free nitrogen of the air. The discussion of the questions involved in such inquiries is outside the scope of the present chapter. From the hygienic standpoint, soil bacteria are chiefly of importance in view of the fact that they can be washed out of the soil into water supplies. An important aspect of this question is the significance of certain bacteriological appearances in a water in relation

to the soil from which it has come or over which it has flowed.

Methods of Examination.—For examination of surface soil or soil near the surface, Houston recommended tin troughs 10 inches by 3 inches, and pointed at one extremity, to be wrapped in layers of paper and sterilised by dry heat. If several of these be provided, then the soil can be well rubbed up and a sample secured and placed in a sterile test-tube for examination as soon as convenient after collection. If samples are to be taken at some depth beneath the surface, then a special instrument, of which many varieties have been devised, must be used. The general form of these is that of a large gimlet-like instrument stoutly made of steel. Just above the point of the instrument the shaft has in it a hollow chamber, and a sliding lateral door in this can be opened and shut by a mechanism controlled at the handle. The chamber being sterilised and closed, the instrument is bored to the required depth, the door is drawn back, and by varying devices the chamber is filled with earth; the door is re-closed and the instrument withdrawn

In any soil the two important lines of inquiry are, first, as to the total number of organisms (usually reckoned per gram of the fresh sample); and secondly, as to the varieties of organisms present. The number of organisms present in a soil is often, however, so enormous that it is convenient to submit only a fraction of a gram to examination The method employed is to weigh the tube containing the soil, shake out an amount of about the size of a bean into a litre of distilled water, and re-weigh the tube. The amount placed in the water is distributed as thoroughly as possible by shaking, and, if necessary, by rubbing down with a sterile glass rod, and small quantities measured from a graduated pipette are used for investigation. For estimating the total number of organisms present in the portion of soil used, small quantities, say 0·1 c.c. and 1 c.c., of the fluid are added to tubes of melted nutrient gelatin or agar; after being shaken, the medium is plated, incubated at 22° C., and the colonies are counted after three or four days. From these the total number of organisms (viable in the medium used) in a given amount of soil can be calculated.

In certain cases it may be necessary to investigate the anaerobic organisms of the soil, e.g. *B. tetani*. The inquiry is necessarily of a qualitative character and the methods to be adopted are those described for the isolation of these organisms (*vide* pp. 97, 641). Information may be acquired by the injection of small portions of the soil into animals (guinea-pigs, mice).

The numbers of bacteria in the soil vary very much. According to Houston's results, fewest occur in uncultivated sandy soils, these containing on an average 100,000 per gram. Peaty soils, though rich in organic matter, also give low results the acidity of such soils inhibiting free bacterial growth. Garden soils yield usually about 1,500,000 bacteria per gram, but the greatest numbers are found in soils which have been polluted by sewage, when the figures may rise to several millions.

Besides the enumeration of bacteria in a soil, an important

question is the types of bacteria present. Frequent soil organisms are *B. mycoides*, *B. subtilis*, and allied types (*vide* p. 505), streptothrix-like organisms, *B. proteus*, and the nitrifying bacteria; but from the public health standpoint it is obviously of more importance to ascertain the presence of organisms indicative of pollution by sewage, *e.g.* typical *B. coli*, *B. welchii*, and streptococci (see p. 891 under Water).

For the detection of these bacteria the following procedures may be recommended:

(*a*) *The B. coli Group* (see Chapter XV.).—A third of a gram of soil is added to 10 c.c. of broth, shaken up, and loopfuls are spread on one or more plates of MacConkey's lactose neutral-red agar. After twenty-four hours' incubation in an inverted position, any red colonies are picked off and subjected to the tests for typical *B. coli*, detailed in Chapter XV., p. 513. If it is necessary to ascertain the actual *B. coli* content of the soil, varying amounts of the soil emulsion are added to tubes of MacConkey's fluid medium, as in water examination (*q.v.*).

(*b*) *The Bacillus welchii* (see Chapter XX.).—To search for this organism 1 gram of the soil is thoroughly distributed in 100 c.c. sterile broth, and of this 1 c.c., 0·1 c c., and 0·01 c.c., are added each to a sterile milk tube. These are heated to 80° C. for ten minutes, and then cultivated anaerobically at 37° C. for forty-eight hours. If the characteristic "stormy clot" reaction is developed, then it may be assumed that this organism is present (*vide* p. 649).

(*c*) *Fæcal Streptococci* (see Chapter VII.).—The best method to employ is that of Prescott and Winslow modified by Mair. This depends on the fact that when *B. coli* and streptococci are growing together in glucose broth, as the medium becomes acid the streptococci tend to outgrow the *B. coli*. If lactose neutral-red agar plates be made at this stage, the colonies of streptococci, being small and intensely red, can be distinguished from the larger colonies of the *B. coli*. They can then be subcultured for investigation. It is evident that here the method must be adopted of taking as a measure of the number of streptococci present, the least quantity of the original fluid in which evidence of their presence can be detected.

We may now give in brief the results obtained by the application of such methods. With regard to the typical *B. coli*, its presence in a soil must be looked on as indicative of recent pollution with excremental matter. The presence of *B. welchii* is also evidence of such pollution, but from the fact that this is a sporing organism the pollution may not have been recent. With regard to the streptococci, on the other hand, on account of their lesser viability outside the animal body, their presence is to be looked on as evidence of recent excremental pollution, as in the case of typical *B. coli*.

While such means have been advanced for the obtaining of indirect evidence of pollution of soil, investigations have also been conducted with regard to the viability in the soil of pathogenic bacteria, especially of those likely to be present in excreta, *e.g.* the typhoid and cholera organisms, etc. The solution of this problem is attended with difficulty, as it is not easy to identify these organisms when they are present in such bacterial mixtures as naturally occur in the soil. Further, bacteria often influence each other's viability in an unfavourable way. For instance, it has been found that the *B. typhosus*, when sown in an organically polluted soil which has been sterilised, can maintain its vitality for several weeks, but if the conditions occurring naturally be so far imitated by sowing it in soil in the presence of soil bacteria, it is found that sometimes the typhoid bacillus disappears in the course of a few weeks, or even in a few days. Further, the character of the soil exercises an important effect on the results; for instance, the typhoid bacillus soon dies out in a virgin sandy soil, even when it is the only organism present. In experiments made by sowing cultures of *V. choleræ* and certain other pathogenic organisms in plots in a field, it was found that after forty days, at the longest, they were no longer recognisable. Further, it is unlikely that strictly pathogenic organisms, even if they remain alive, can multiply in soil under natural conditions. In the case of a sporing organism such as the *B. anthracis*, the capacity for remaining in a quiescent condition of potential pathogenicity is, of course, much greater.

Water

In the bacteriological examination of water three lines of inquiry may be followed. First, the number of viable bacteria per cubic centimetre may be estimated. Second, the kinds of bacteria present may be investigated. Third, it may be necessary in the case of a particular organism, if present, to ascertain in what number per c.c. it occurs.

Methods.—*Collection of Samples.*—Samples are best collected in 8-ounce sterile stoppered bottles.

In the case of water taken from a house tap, the water should be allowed to run for some time before the sample is taken, as water standing in pipes in a house is under very favourable conditions for bacterial multiplication.

With river waters it is best to immerse the sampling bottle and then remove the stopper with forceps. Care must be taken not to touch the river bed, as the vegetable matter covering it contains many organisms. When water has to be taken from below the

surface of a well or lake, a weighted sample bottle must be used. Several special bottles have been devised for such a purpose. Quite good results are obtained by tying two short lengths of string to the neck and stopper of an ordinary bottle respectively; any required length of string can afterwards be joined to these. A piece of lead is attached to the bottom of the bottle by wires passing round the neck. The whole is then wrapped in paper and sterilised. For use the bottle is carefully lowered to the required depth by the string attached to the neck, the stopper is jerked out by means of the other string, and the bottle filled.

The bottles should be packed in sawdust and ice and the primary inoculations made as soon as possible. Otherwise the bacterial content may alter, and an erroneous idea of the number present will be obtained. Immediately after collection a slight diminution in numbers may be observed, but at any rate after six hours an increase over the initial numbers is manifest.

Counting of Bacteria in Water.—This is done by adding a given quantity of water to 10 c.c. of liquefied agar, plating, and counting the colonies which develop. The amount of water added depends on its source, and varies from 0·1 c.c. of a water likely to have a high bacterial content to 5 c.c. of a purer water. The plates should be duplicated and one incubated at 37° C., the other at 22° C. The medium should be standardised to pH 7·2. It has been customary in the past to use a gelatin medium for low temperature incubation, but it is found more convenient to employ nutrient agar throughout. The plates incubated at 22° C. give an idea of the numbers of purely saprophytic bacteria present; the plates incubated at 37° C., those of more parasitic nature. As the intestinal bacteria grow at 37° C., the determination of the numbers at this temperature is important. The counts on the two media usually differ according to the prevalence of saprophytic or parasitic organisms respectively. In the case of the plates at 37° C. usually forty-eight hours' incubation is allowed before the colonies are counted, but the plates at the lower temperature should be incubated longer before counting to facilitate recognition of the colonies.

Detection of the Presence of Special Organisms.—(*a*) *The B. coli Group*.—In ordinary public health work, it may be taken that the most frequent and important inquiry with regard to a water is directed to the investigation of the presence or absence of the *B. coli* and its congeners. Here the method which has usually been adopted is to determine the smallest quantity of a water which gives evidence of containing organisms of this type.

As the primary culture medium MacConkey's bile-salt lactose peptone water[1] (with litmus or neutral-red added) is used. In this medium the typical members of the *B. coli* group produce acid and gas. It is thus necessary to put the medium into bottles containing an inverted tube to indicate gas production (*vide* p. 80). Suitable containers are 2-ounce cylindrical medicine bottles 4½ inches high by 1¼ inches in diameter stoppered with cotton wool. The following quantities of the water are added to a series of such bottles: 50 c.c., 20 c.c., 10 c.c., 5 c.c., 1 c.c., and it may be in specially suspicious waters, 0·5 c.c., 0·1 c.c., and even 0·01 c.c. It is advisable to

[1] Two per cent. peptone water with 0·25–0·5 per cent. sodium taurocholate and 1 per cent. lactose (*vide* p. 73).

duplicate the series. The quantity and concentration of the medium in each bottle is so arranged that, when the sample is added, the resulting fluid shall be of the concentration of MacConkey's medium as ordinarily prepared. Thus, the bottle to which the 50 c.c. sample is to be added contains 10 c.c. of a six-fold concentration of the medium. For the 20 c.c. sample, 20 c.c. of a medium of double strength ; for 10 c.c., 10 c.c. of the double strength medium ; and for 5 c.c., 5 c.c. of double strength medium. For smaller samples, 5 c.c. of the ordinary MacConkey's medium may be used.

For each sample it is necessary to have sterile 25 c.c., 10 c.c. (graduated to tenths), and 1 c.c. (graduated to hundredths) pipettes.

The bottles are incubated for forty-eight hours, and it is well to read the results at the end of the first twenty-four hours also. The formation of acid and gas in a bottle is generally recognised as " presumptive evidence " of the presence of members of the *B. coli* group, but it is usual to investigate further the bacteria giving rise to this change and determine whether they are " typical " or " atypical " *B. coli*. With this end in view, each bottle in which acid and gas are present is shaken, two or three loopfuls are placed on a plate of MacConkey's neutral-red bile-salt lactose agar and spread over the surface as in making successive stroke inoculations. The plates are incubated for twenty-four hours. As typical *B. coli* produces acid in lactose any colonies of such an organism are of a rose-red colour. Representative colonies are then subcultured on sloped agar and used for the further tests (*vide* p. 512) ; gelatin liquefaction, indole formation, fermentation of adonitol and inosite, the Voges-Proskauer and methyl red reactions and the citrate-utilisation test.

It is well in dealing with the neutral-red lactose agar plates to inoculate lactose peptone waters tube from all the kinds of colonies present, whether these are red or not, as sometimes an organism which is really a lactose fermenter does not produce a red colour on the solid medium.

The object of growing suspicious colonies on a range of media such as that given, is to enable typical *B. coli* to be recognised when present. This subject has been more fully discussed in Chapter XV. The characters of a typical *B. coli* on which, in our opinion, special stress should be laid are : absence of gelatin liquefaction, fermentation (with acid and gas production) of lactose, absence of fermentation of inosite, production of indole from peptone water and a positive methyl-red reaction.

The final result is stated in terms of the smallest amount of the sample which contains typical *B. coli*, provided both bottles (in the duplicate series) inoculated with this amount yield the organism and all larger volumes tested also show it. It must be remembered that the method is one of random sampling and irregular results may sometimes be obtained, *e.g.* a certain volume may yield *B. coli* when the organism appears to be absent from a larger quantity.[1]

(*b*) *B. welchii and streptococci*.—The methods for the detection of these organisms are those which have already been given (p. 889).

[1] For further information on the methods of bacteriological examination of water supplies, reference should be made to a recent report by the Ministry of Health (*Report on Public Health and Medical Subjects*, No. 71, H.M. Stationery Office, 1936).

The detection of *B. welchii* has been used in water examination as a means of proving the presence of excretal pollution. Fifty c.c. of water are added to 100 c.c. of sterile milk in a stoppered bottle, and after the mixture has been heated at 80° C. for ten to fifteen minutes a layer of sterile liquid paraffin is superimposed to produce anaerobic conditions. The mixture is then incubated at 37° C. for twenty-four to forty-eight hours and observed for the " stormy-clot " reaction.

Wilson and Blair devised a method for estimating the number of *sporing anaerobes* in water. The medium is nutrient agar (3 per cent agar), 100 c.c. ; 20 per cent. glucose, 5 c.c. ; 20 per cent. sodium sulphite, 10 c.c. ; and 8 per cent. ferric chloride, 1 c.c. To 40 c.c of the medium 40 c.c. of water are added and the mixture is poured into a large Petri dish After solidifying, it is covered with a layer of 20 c.c. of the same medium mixed with an equal volume of sterile water. *B. welchii* and other sporing anaerobes produce black colonies due to the reduction of the sulphite and the production of sulphide of iron According to Wilson and Blair a very pure water should show no black colonies and a potable water should yield not more than four such colonies. The medium has been modified successfully by substituting anhydrous ferrous sulphate for ferric chloride.

The *streptococci* present in polluted water are of the enterococcus type (*vide* p. 291). The test for their presence should be quantitative like that for *B. coli*. Varying amounts of water are added to 1 per cent. glucose broth which is incubated for forty-eight hours, when the presence of streptococci can be determined by microscopic examination of the culture medium, *e.g.* by hanging-drop preparations, or by a plating method as described on p. 889

Water derived from any natural source contains bacteria, though, as in the case of some artesian wells and some springs, the numbers may be very small, *e.g.* 4 to 100 per c.c. In rain, snow, and ice there are often great numbers, those in the first two being derived from the air. Great attention has been paid to the bacterial content of wells and rivers. With regard to the former, precautions are necessary in arriving at a judgment. If the water in a well has been standing for some time, multiplication of bacteria may give a high numerical count. To meet this difficulty the well ought, if practicable, to be pumped dry and then allowed to fill, in order to ascertain the bacterial content of the water entering the well. Again, if the sediment of the well has been stirred up, a high bacterial count is obtained. Ordinary wells of medium depth contain from 100 to 2000 per c.c. With regard to rivers, very varied results are obtained. Moorland streams are often fairly pure. In an ordinary river the numbers present vary at different seasons of the year, while the prevailing temperature, the presence or absence of decaying vegetation, or of washings from land, and dilution with large quantities of pure spring water, are other important factors.

Thus the Franklands found the rivers Thames and Lea purest in summer, and this they attributed to the fact that in this season there is most spring water entering, and very little water as washings off land. In the case of other rivers bacteria have been found to be fewest in winter. A great many circumstances must therefore be taken into account in dealing with mere enumerations of water bacteria, and such enumerations are only useful when they are taken simultaneously over a stretch of river, with special reference to the sources of the water. Thus it is usually found that immediately below a sewage effluent the bacterial content rises, though in a comparatively short distance the numbers may markedly decrease, and it may be that the river as far as numbers are concerned, may appear to return to its previous bacterial content. The numbers of bacteria present in rivers flowing through inhabited districts therefore vary greatly.

The bacterial count is of great use in estimating the efficacy of the filter-beds of a town water-supply. These usually remove from 95 to 98 per cent. of the bacteria present, and a town supply as it issues from the filter-beds should not contain more than 100 bacteria per c.c. Again, it is found that the storage of water brings about a very marked purification. Thus Houston showed in one series of observations that while 93 per cent. of samples of raw river Lea water contained *B. coli* in 1 c.c. or less, in the stored water 62 per cent. of the samples showed no *B. coli* to be present in 100 c.c. According to Coplans, however, the diminution is not necessarily due to the organisms being killed; the real cause may be the agglutination of the bacteria following on changes in the electrical conductivity which take place in stored water.

Much more important than the mere enumeration of the bacteria present in a water is the question whether these include forms pathogenic to man. The most important of these are the typhoid-paratyphoid group, *B. dysenteriæ*, and, in certain circumstances, the cholera vibrio. On account of the small numbers which may be present in a dangerous water, the direct isolation of these organisms is often impracticable and in any case unnecessary for the condemnation of an unsafe water supply. For public health purposes we therefore seek for the presence of indirect bacteriological evidence which might point to the contamination of a water by human excreta. If this be found we deduce that the water is dangerous, as organisms from any case of intestinal disease occurring in the catchment area may find access to it. The criterion here adopted is the determination

of the numbers of typical *B. coli* present in the water. Klein and Houston have pointed out that, in crude sewage, members of the coli group are practically never fewer than 100,000 per c.c. In these circumstances the presence of typical *B. coli* in a water is the best indirect evidence of the possibility of disease organisms of intestinal origin being likely to gain access to that water. It must, however, be clearly recognised that these organisms are only as it were indicators of excretal contamination.

The difficulty, however, is that (except in the case of water from artesian wells) if a sufficient quantity be taken, evidence of the presence of *B. coli* will always be found. This arises from the fact that this organism is present also in the excreta of birds and other animals, and it is impossible in the present state of knowledge to distinguish between organisms coming from these different sources. Thus even in the moorland waters so much used for urban supplies, there may be a fairly high content of *B. coli*—without the least evidence of these being derived from human sources, and the consumption of such a water, even in an unfiltered condition, may be perfectly safe. It is impossible to set up any absolute standards of the bacteriological purity of a water based only on the estimation of the numbers of *B. coli* present. In any particular case the results must be considered along with those of chemical analysis and inspection of the locality. The difficulty is greatest when dealing with water derived from sewage-contaminated rivers, from agricultural land, and from surface wells. With regard to the first two sources, the water should never be used in an unfiltered condition, and with regard to the last, every case must be considered on its own merits. In addition to filtration, chlorination of such waters has come to be a usual practice. It may be said that under ordinary circumstances an inspection of the surroundings and an unfavourable chemical analysis are sufficient to condemn a water, even if a bacteriological examination showed the absence of *B. coli* in large quantities; and further, if in a suspicious locality the bacteriological analysis yielded a bad result, the water ought to be condemned even when from the chemical analysis it could be passed. If any general standard is to be applied it is probably safe to say that when typical *B. coli* is found in 10 c.c. or less of a water from a suspicious locality it is unsafe for human consumption. On the other hand, waters of a high degree of purity show absence of *B. coli* from 50 c.c. or even 100 c.c., and it may be said that in a pure water *B. coli* should be absent from 25 c.c.

The examination for the presence of *B. coli* finds a further application in determining the efficiency of a filtration process, and here it is extraordinarily delicate. While again it is difficult to lay down an absolute standard of purity, the filtration methods in use are, if properly worked, capable of delivering an effluent which does not yield *B. coli* in amounts less than 100 c.c., and such a degree of efficiency should in all cases be aimed at.

As *B. coli* is fairly widespread in nature, valuable supporting evidence is found in the presence of *B. welchii* and of streptococci, both of which are constant inhabitants of the human intestine. The spores of the former are numerous in sewage, and the presence of the latter can always be recognised in 0·001 gram of human fæces. The significance attached to the presence or absence of these organisms has already been referred to on p. 889.

It should be emphasised that in water artificially polluted with sewage containing intestinal bacteria, these can be detected by bacteriological methods in mixtures from ten to a hundred times more dilute than those in which the pollution can be detected by purely chemical methods.

The Isolation of the Typhoid-Paratyphoid Group from Water.—Though the typhoid bacillus has been isolated, on occasions, from polluted waters responsible for outbreaks of enteric fever, the technical difficulty of its isolation from a water in which it is likely to be present, at the most only in very small numbers and also along with a greater number of other organisms, renders the procedure hardly practicable as a routine method. In attempting to isolate this organism, it is necessary to utilise some method of concentrating or enriching it in the specimen, and a relatively large quantity of water must be tested for the purpose. Various methods have been used. Of these the following may be recommended :

Enrichment by brilliant green.—The general principle of this method is referred to on p. 74. To 900 c.c. of the water in a stoppered sterile flask is added 100 c.c. of a 10 per cent. sterile solution of peptone, 5 grams of sodium chloride, and 5 c.c. of a 1 : 1000 solution of brilliant green in distilled water. In this way the water is converted into a suitable culture medium and the brilliant green, in virtue of its selective action, tends to enrich organisms of the typhoid-paratyphoid group while suppressing *B. coli*. After incubation for twenty-four hours, sub-inoculations are made on MacConkey plates and suspicious colonies investigated by the procedure detailed on p. 555. Subinoculations may also be made after forty-eight hours' incubation.

The Isolation of Vibrio Choleræ from Water.—For this purpose, advantage is taken of the enrichment of this organism when growing in an alkaline peptone solution : 900 c.c. of the suspected water, to which have been added 100 c.c. of a sterile 10 per cent. solution of peptone (adjusted to pH 9·0) and 5 grams of sodium

chloride, are distributed in stoppered sterile Erlenmeyer flasks so that each flask contains only a shallow layer of the mixture. The flasks are incubated at 37° C., and if vibrios grow they may be isolated and identified by the methods described on p. 576.

SEWAGE

It is sometimes necessary to examine the bacterial content of sewage, especially in connection with the efficiency of purification works. The main lines of inquiry are here the same as for water, the only modification necessary being that, in consequence of the high bacterial content, much smaller quantities of the raw material must be worked with, *e.g.* a series of decimal dilutions of the sewage. With regard to the numbers of bacteria in sewage, these may vary from a million to ten millions or even more per c.c., and here of course the question of the presence of intestinal organisms of the *B. coli* group is of importance. The numbers of these are large, and members of the group may be detected in 0·000001 c.c. or less. Recently it has been shown that by selective or enrichment methods (*vide* p. 74) organisms of the typhoid-paratyphoid group can be isolated from communal sewage (Wilson ; Gray, and others). The numbers of *B. coli* present are frequently considerably reduced by purification methods, but it is to be noted that, even when such methods are most successful, this organism may yet be present in considerable numbers. By no purification method has the production of a potable water been attained, and the high content of *B. coli* in effluents suggests that the passage of typhoid bacilli through a purification system is possible.

The part which bacteria play in the purification of sewage constitutes a question of great interest, to which much attention has been directed.

Various methods have been adopted for the biological purification of sewage. One of the earliest methods to be used was as follows : the sewage coming from the mains was run on to a bed of gravel, clinker, or coke, on which it was allowed to stand for some hours. The effluent was then run out through the bottom of the bed, which was allowed to rest for some hours before being recharged. In a modification of this method the sewage after treatment in a closed tank (" septic tank ") is allowed to percolate slowly through a bed consisting of large porous objects, such as broken bricks or large pieces of coke, and here the percolation may be constant, no interval of rest being given. In the explanation given of the rationale of this process, sewage is looked on as existing in three stages. (1) First of all, *fresh sewage*—the newly mixed and very varied material as it enters the main sewers. (2) Secondly, *stale sewage*—the ordinary contents of the main sewers. Here

there is abundant oxygen, and as the sewage flows along, there occurs by bacterial action formation of carbon dioxide and ammonia, which combine to form ammonium carbonate. This is the sewage as it reaches the purification works. Here a preliminary mechanical screening is adopted. Sedimentation is also frequently employed at this stage, and in this way 50 per cent. of the suspended matter can be separated out as " sludge." After this the sewage is run into an airtight tank—the septic tank. (3) It remains there for from twenty-four to thirty-six hours, and becomes a foul-smelling fluid—the *septic sewage*. The chemical changes which take place in the septic tank are of a most complex nature. The sewage entering it contains little free oxygen, and therefore the bacteria which flourish in the tank are probably largely anaerobic, and the changes which they originate consist of the formation of comparatively simple compounds of hydrogen with carbon, sulphur, and phosphorus. As a result, there is a great reduction in the amount of albuminoid nitrogen, and of solid organic matter. The last is important, as the clogging of ordinary filter-beds is largely due to the accumulation of such material, and particularly matter consisting of cellulose. One further important effect is that the size of the particles of the deposited matter is decreased, and therefore it is more easily broken up in the next stage of the process. This consists in running the effluent from the septic tank on to filter-beds, *e.g.* of coke, where a further purification process takes place. By this method there is first an anaerobic treatment, succeeded by an aerobic ; in the latter the process of nitrification occurs by means of the special bacteria concerned. The results are of a satisfactory nature, and there is often a marked diminution in the number of coliform organisms present.

In the earlier stages of any sewage purification, there is little doubt that the organic material present is being split up by ordinary putrefactive bacteria. In the mains and where open systems of purification are at work, aerobic forms play the chief part, while in the closed methods anaerobic organisms are those chiefly concerned. In contact and percolating systems there is evidence that at first the purifying action of bacteria is materially furthered by physical processes. Thus Dunbar showed that when such a substance as coke is used in a sewage filter-bed a considerable amount of the protein material is removed in a very few minutes by adsorption ; being of a colloidal nature, it is readily deposited under such circumstances in the pores of the coke in the form of films. After a time such a filter-bed becomes clogged, but on access of oxygen being allowed, it regains its adsorptive properties—probably from the oxidation of the material adsorbed.

During the purification of sewage, various processes are at work : First, the action of ordinary bacteria splitting up complex organic matter ; secondly, the action of nitrifying bacteria building up nitrates from ammoniacal products ; thirdly, the action of denitrifying bacteria which reduce nitrates to free nitrogen (the presence of which in filter-beds can be demonstrated) ; fourthly, the action of higher forms of vegetable life and possibly of certain low forms of animal life ; fifthly, it is possible that direct chemical oxidation of the earlier products of bacterial action may occur, and in any case the access of an abundant oxygen supply to adsorbed material hastens its destruction.

The Activated-Sludge Process—In recent years the activated-sludge process has come into vogue and has already proved to be an efficient and economic method of sewage purification. Raw sewage is run into tanks in which it is mixed with 15 to 30 per cent. "activated sludge" (derived from previous treatment of sewage) and thoroughly aerated either from a compressed air supply or by mechanical agitation; thereafter it is passed into sedimenting tanks and the sludge allowed to separate. This sludge is practically odourless and can be used as a fertiliser for agricultural purposes or is allowed to undergo anaerobic fermentation as a source of power-gas. Purification by this process is remarkably rapid, occurring in a few hours. While the principles underlying the method are not completely understood, bacterial action plays an important part. The sludge derived from previous treatment of sewage supplies a suitable bacterial inoculum for the raw sewage. The conditions of treatment are aerobic and presumably the purification is oxidative in nature and biologically similar to that which occurs in the aerobic contact-beds of the older processes (*vide supra*). Physical factors are also concerned and the colloidal material undergoes precipitation and sedimentation.

The effluent from a sewage purification system may contain as many bacteria as the sewage entering, but there is often a marked diminution. It is said by some that pathogenic bacteria do not live in sewage. The typhoid bacillus has been found to die out when placed in sewage, but it certainly can live in this fluid for a much longer period than that embraced by any purification method. Thus the constant presence of *B. coli* and other intestinal organisms which has been observed in sewage effluents must here still be looked on as significant. Sometimes the effluent is disinfected by chlorination before being discharged into a river.

Milk

The bacteriology of milk presents two aspects, the economic and the hygienic, with the latter of which this chapter is mainly concerned. It may be said that the bacteriological condition of ordinary market milk, as sold in the large communities of this country, is far from satisfactory from the hygienic standpoint, and the hygienic aspects of milk bacteriology are therefore of special importance in preventive medicine. In the bacteriological sense, the ordinary raw milk is perhaps the most impure of foods, and therefore a potential source of infection. Primarily, cow's milk is a sterile fluid, but from the time it flows along the large ducts of the udder, which may contain bacteria of various types, it becomes progressively contaminated. It is an excellent culture medium, especially for intestinal organisms, and any contamination is therefore progressive unless at very low temperatures. The main sources of the bacteria always found in fresh milk are the external surface of the udder, the hands of the milkers, utensils in which the

milk is collected, and often dust that has gained access to the milk; but under unhygienic conditions of collection and distribution large numbers may be added by gross pollution. Under the most favourable conditions, fresh milk may contain about five hundred organisms per c.c., but the bacterial content may be greatly increased under unhygienic conditions, and, on the other hand, it has been shown that the numbers of bacteria may be easily controlled by attention to the cleanliness of the cowhouse, by grooming of the animals, washing the udder before milking, etc. There is some evidence that for a short time after milk is withdrawn a slight diminution of the bacterial content may take place, but before it reaches the consumer, especially in city supplies and in warm weather, the bacterial content of apparently fresh milk may rise to several hundred thousands, or even millions per c.c. The organisms present are mainly intestinal bacteria, *e.g.* the *B. coli* group, lactobacilli, sporing aerobes and anaerobes, and streptococci, and this is specially significant of the nature of the contamination to which milk is subjected in the process of collection.

The Souring of Milk.—Under ordinary conditions the first evidence of bacterial activity, and from the economic standpoint the most important, is the occurrence of souring due to the formation of lactic and other allied acids, and the action of these on the albuminous constituents is one of the factors in curdling. The subsequent changes vary with the bacteria present, but ultimately these lead up to putrefaction of the ordinary type. The importance of the souring of milk has caused much attention to be devoted to the process, and a variety of bacteria have been described by various observers.

Three main types occur. (*a*) The first of these is the *Streptococcus lacticus* (*S. lactis*), originally described by Kruse. It is now recognised that this organism is practically identical with the enterococcus (*vide* p. 291).

(*b*) *The lactic acid bacilli* (*Lactobacilli*) are a group of organisms certain of which occur normally in the intestine of various mammals and are specially numerous in the young animals before weaning. They are non-sporing, non-motile, Gram-positive organisms, non-liquefiers of gelatin; they show great pleomorphism, and the same strain may present marked differences in microscopic appearance according to the conditions under which it is growing. They are highly resistant to acid, and some will grow in the presence of 1 per cent. acetic acid; on this account they are called *acidophilic* or *aciduric*. Some are thermophilic and flourish at 50° C. Among these organisms *B. acidophilus* and *B. bifidus* may be specially mentioned as occurring in the human subject. No pathogenic properties have been attributed to these bacteria; but a related organism, *B. acidophilus odontolyticus*, has been described by

McIntosh and his co-workers as the cause of dental caries. The Boas-Oppler bacillus, another representative of the group, is found in the stomach contents in states in which HCl is absent, *e.g.* gastric cancer, and lactic fermentation is occurring. Döderlein's vaginal bacillus also belongs to this group.

B. acidophilus is a rather long, stout bacillus (1 μ or more broad), with a tendency to curl at the ends ; but a more slender form which tends to be in chains also occurs (Fig. 209). The organism may be recovered from fæces by plating in 1 per cent. peptone agar containing 2–4 per cent. lactose, or, better, in whey agar [1] or after preliminary enrichment in neutral broth to which 1 per cent. acetic acid has been added, and incubation at 37° C. The colonies are very minute, and for their detection one must examine the culture under the low power of the microscope ; they are of two types, the one a delicate feathery growth (Fig. 210), the other rounded

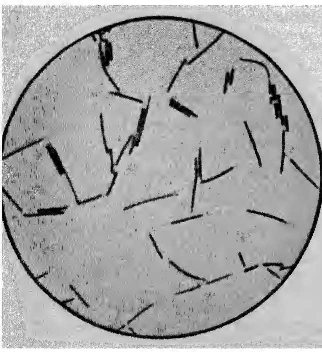


FIG. 209. — *B. acidophilus* from twenty-four hours' growth on agar. Gram's method. ×1000.

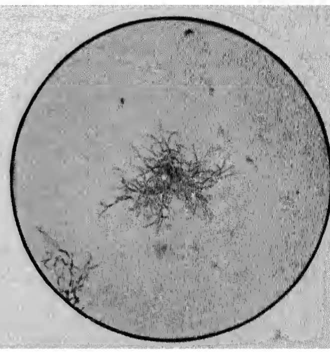


FIG. 210.—Surface colony of *B. acidophilus* on agar plate; twenty-four hours' growth. ×100.

or fusiform and often with fringe-like projections (" crab colonies "). *B. bulgaricus* is a related organism ; it has the same microscopic characters and forms similar feathery colonies ; it can be differentiated from *B. acidophilus* by the fact that the latter forms acid in 1 per cent. maltose broth in forty-eight hours at 37° C., whereas *B. bulgaricus* fails to do so ; the latter also does not flourish in the human intestine. Both these organisms are aerobes.

B. bifidus is the predominant organism in the intestine of young breast-fed infants, and in a film of the fæces it appears in practically pure culture (Fig. 211), being 4–5 μ long by 0·7 μ broad, and straight or slightly curved, often with bulbous ends ; but in cultures pleo-

[1] Whey broth is prepared by adding to skimmed milk at 80°–90° C. just sufficient 10 per cent. HCl to precipitate the casein. The fluid is filtered through cotton wool and then through paper and the pH adjusted to 6·8–7·0 with NaOH. Finally, 0·5 per cent. peptone is added and the mixture autoclaved at 120° C. for fifteen minutes and filtered ; whey agar consists of the above medium *plus* 1·2 per cent. agar.

morphism is marked (Fig. 212). Often the bacilli are only weakly Gram-positive. In order to obtain this organism in culture special conditions are required. Deep tubes of 1 per cent. lactose or glucose broth neutral to litmus, to which a small piece of sterile rabbit's kidney has been added, are heavily inoculated with a suspension of the fæces and sealed with a layer of sterile vaseline and are kept at 37° C. for six to eight days. Any gas which forms at first is expelled by re-melting the vaseline. Stroke cultures are then made on plates of 1 per cent. glucose agar or Löffler's serum and are incubated anaerobically. After forty-eight hours *B. bifidus* has formed greyish pin-head colonies resembling those of diphtheroids, which in the next day or two enlarge to 3–4 mm. in diameter (the only other organisms likely to be present are enterococci which form larger, whitish colonies). Rich subcultures

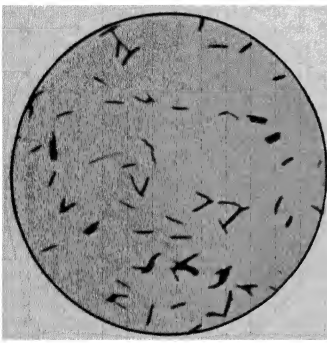


FIG. 211.—*Bacillus bifidus*, as seen in film preparation from fæces of an infant. Gram's method. ×1000.

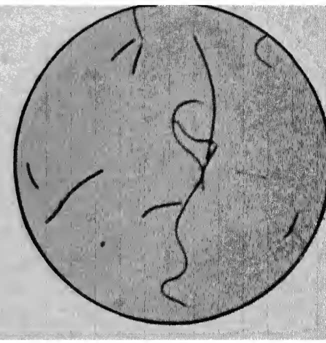


FIG. 212.—*Bacillus bifidus*, from a three-days' culture on agar. Gram's method. ×1000.

on glucose agar and in glucose broth will now be found to grow aerobically.

The above-mentioned organisms do not form indole and fail to produce gas from carbohydrates. A number of similar organisms have been isolated from fermenting milk or carbohydrate materials, some of which are gas producers.

(*c*) The third group of milk-souring organisms is the *B. coli group*, certain types of which were originally described in connection with this process (*vide* p. 507). These organisms produce both acid and gas from lactose, and curdling of milk by their growth may be associated with gas formation.

As already stated, there occur in milk a number of bacteria of very different morphological and cultural characters with the common capacity of producing lactic and other acids, and the special qualities of any souring process probably depend on the particular combination of bacteria present. There is

considerable evidence that the occurrence of souring holds in abeyance for a time the activity of putrefactive organisms whose special characteristic is the disintegration of the protein molecules. Many changes, which may be denominated economic diseases of milk, are also due to bacteria, *e.g.* the occurrence of ropy milk, bitter milk, and coloured milk.

Pathogenic Organisms in Milk.—From the hygienic standpoint the most important consideration is that of the conditions under which pathogenic organisms gain access to milk. These may originate in diseased conditions occurring in the cow, or the milk may become contaminated from cases of human disease. With regard to the former, the two most important are inflammatory and suppurative disease of the udder, and tuberculosis. Thus chronic mastitis is prevalent and is due to streptococci (*S. agalactiæ*, *vide* p. 292). The milk in such a case may contain pus and blood-stained serum along with large numbers of these organisms. Such milk finds its way into large milk supplies, but it is doubtful if it is responsible for active disease in man as the common types of mastitis streptococci are probably non-pathogenic to the human subject. Some observers claim, however, that cases of acute bovine mastitis are due to hæmolytic streptococci of human origin. Thus persons harbouring virulent streptococci in their throats may by contact with cows, *e.g.* in milking, infect the udders; the resulting mastitis may in turn lead to contamination of the milk and spread the infection widely among other persons. It is supposed that scarlatina may also be spread in a similar way by milk. *B. melitensis* may occur in the milk of infected goats, and undulant fever is usually traceable to this source (see Chapter XIX.). *B. abortus*, which is closely related to this organism, may be present in the milk of infected cows, and the question of its pathogenicity to the human subject has been discussed (*vide* p. 622). Human cases of foot-and-mouth disease have also been recorded and traced to milk from infected animals. In outbreaks of bacterial food poisoning due to the *Salmonella* group, cow's milk has been found to be infected with organisms of this type and has been responsible for the condition (*vide* p. 540). Cases have been recorded of "food-poisoning" due to milk and milk products containing staphylococci (*vide* p. 282). The possibility of this originating from staphylococcal infection of the cow's udder must be borne in mind.

Tuberculosis in the cow is a serious source of human tuberculosis arising from the consumption of milk. It should be noted

that the disease is exceedingly prevalent in milch cows in this country. The relation of the bovine type of the tubercle bacillus to the human is discussed in Chapter X. Here it need only be said that where tuberculous disease occurs in the cow's udder, tubercle bacilli will be found in the milk, and, further, that where generalised tuberculosis occurs in the animal, tubercle bacilli have been found in the milk without evidence of the udder being diseased. The importance of this observation is evident from the fact that a cow with large deposits of tubercle in the lungs and peritoneum may to external inspection appear in normal condition. Further, under conditions of unhygienic milking, tubercle bacilli may gain access to milk from the excreta of infected animals and by contamination of the milk from the air, dust, and filth of a cowhouse occupied by tuberculous cattle. Though udder tuberculosis occurs in only 0·2 per cent. of dairy cows, and though only a very small proportion of milk samples from individual cows contain tubercle bacilli, the mixing of milk for market purposes increases the percentage of specimens of tuberculous milk as supplied to the community. This percentage may be high, *e.g.* 8 to 17 per cent. in certain of the large communities.

Apart from infective conditions of the cow itself, milk may be a disseminating agent through handling by those suffering from disease. Two diseases occasionally spread in this way are diphtheria and enteric fever. In the former case the bacilli have been actually isolated from the milk. In the case of typhoid fever the chief danger lies in the milk being contaminated by a "carrier" (see Chapter XV.). In the same way milk may be a source of choleraic and dysenteric infection in countries in which these diseases are prevalent. There is good reason to believe also that cases and outbreaks of sore throat and scarlatina may owe their origin to milk infected with a hæmolytic streptococcus from a person handling the milk while harbouring this organism, *e.g.* a convalescent or an unrecognised case. Similarly a contaminated water used for "watering" milk has been known to give rise to milk-borne outbreaks of enteric fever. House-flies infected with organisms of the typhoid, paratyphoid, and dysentery groups (*vide* p. 529) may contaminate milk with these organisms, and the same applies to the cholera vibrio in countries in which cholera is epidemic.

A very important bacteriological question is the part played by milk in the causation of infantile diarrhœa. It has been long recognised that cow's milk is responsible for a considerable

morbidity and mortality among infants from acute diarrhœa, especially during the summer months when, as might be expected, the bacterial content of milk is generally highest; and without considering any specific bacterium as the causal agent, it might be supposed that the ordinary flora of contaminated milk in excessive numbers or the chemical products resulting from their growth, are responsible for such diarrhœal conditions in infants. Specific organisms have, however, been noted in cases and outbreaks of infant diarrhœa, e.g. *B. dysenteriæ*, B. Morgan No. 1 (*vide* p. 553), and milk cannot always be directly incriminated.

Bacteriological Standards and Graded Milks.—Bacteriological standards have now been extensively applied in controlling milk supplies. It may be said that with proper precautions as regards the hygienic collection and distribution of milk, the bacterial content should not exceed 30,000 viable organisms per c.c. The actual factors concerned in reducing bacterial contamination to a minimum need not be detailed here. For practical information on this subject the reader is referred to works on hygiene.

In recent years, with a view to ensuring that supplies of cow's milk as free as possible from danger to health should be available to the public, grades of milk have been recognised and sold as such, while standards have been laid down for controlling these graded milks. Thus, in the case of the so-called *certified milk*, the bacterial content should not exceed 30,000 per c.c. and *B. coli* should be absent from 0·1 c.c.; this milk must be from tuberculin-tested cows and no heat must have been applied to it.

The following standards have been laid down under the Milk (Special Designations) Orders (1936) of England and Scotland respectively:

England.—*Tuberculin tested* and *Accredited milks* must not decolorise methylene-blue (according to a technique prescribed—*vide infra*) within four and a half hours during the period May to October, and within five and a half hours during the period November to April. Coliform bacilli must be absent from 0·01 millilitre.[1]

Tuberculin tested (pasteurised) milk must not contain more than 30,000 bacteria per millilitre.

Pasteurised milk must not contain more than 100,000 bacteria per millilitre.

Scotland.—*Certified* and *Tuberculin tested (pasteurised) milks* must not contain more than 30,000 bacteria per millilitre and coliform bacilli must be absent from 0·1 millilitre.

Tuberculin tested and *Standard milks* must not contain more than 200,000 bacteria per millilitre and coliform bacilli must be absent from 0·01 millilitre.

Pasteurised milk must not contain more than 30,000 bacteria per millilitre.

[1] The millilitre may be taken as equivalent to the cubic centimetre.

The Sterilisation and Pasteurisation of Milk.—The danger arising from milk being contaminated by disease organisms has caused much attention to be paid to the subject of their destruction before the milk is consumed. The only feasible method is sterilisation by heat, and it is fortunate that practically all the important organisms to be considered are non-sporing forms and thus are easily destroyed. To obviate the development of the rather unpleasant taste caused by boiling milk, Pasteur's method of heating the milk for twenty minutes at 60° to 80° C. has been extensively used. This usually kills all but about 5 per cent. of the organisms present, and will destroy the hæmolytic streptococci, the tubercle bacillus, *B. abortus*, typhoid-paratyphoid bacilli and *B. diphtheriæ*. Sporing putrefactive forms, however, often survive, and unless the pasteurised milk be rapidly cooled, the action of the process as an economic preservative is largely nullified, more especially as the protective milk-souring forms are destroyed. The boiling of milk for two or three minutes will kill all harmful organisms, and although some spores may survive, this is by far the most simple and practical method of disinfection for domestic application. Boiling has been objected to, however, on account of the destruction of vitamins, *e.g.* the anti-scorbutic principle. and if very young children on an exclusively milk diet be given boiled milk alone, in a certain number of cases scurvy results.

Pasteurisation at a low temperature has now been generally accepted for the disinfection of milk, and this process has been adopted for commercial purposes and recognised by the Health Authorities under certain conditions, *e.g.* the "Holder" method in which the milk is maintained at a temperature of 62·8°–65·5° C. (145°–150° F.) for thirty minutes, and then immediately cooled. The bacteriological standards for pasteurised milk have been given above. It is doubtful whether pasteurisation on a large scale for commercial purposes is uniformly effective, and this is borne out by the bacteriological examination of specimens of pasteurised milk sold as such. The question has also been raised as to whether, in the process of commercial pasteurisation, there is a sufficient margin of safety as regards the destruction of the tubercle bacillus. The thermal death-point of the tubercle bacillus in milk has been investigated by various workers. It has been shown that milk containing large numbers of tubercle bacilli (artificially and naturally infected) is rendered non-pathogenic (on inoculation into guinea-pigs) by exposure for thirty minutes at 60° C., and that an infected milk passed through a commercial pasteurising plant is also disinfected in the same way. The officially recognised method of commercial pasteurisation would thus appear to allow sufficient margin of safety as regards the destruction of the tubercle bacillus, provided the pasteurising apparatus is operated with reasonable care.

Methods of Examination.[1]—As regards the enumeration of the total viable bacteria in milk and the estimation of the *B. coli* content, the methods used in water examination (*q.v.*) may also be applied. Decimal dilutions, *e g* 1 : 10, 1 : 100, 1 : 1000, 1 : 10,000 —are made with sterile water in sterile bottles. 1·0 c.c. of each of these is then plated in standardised nutrient agar, and the plates are incubated at 37° C. for two days. In this way the number per c.c. can be calculated. The *B. coli* content can be estimated by adding varying amounts to MacConkey's fluid medium in Durham tubes, as in water examination, *e.g.* 1 c.c. of each of the dilutions referred to above. Acid with gas production is presumptive of the presence of *B. coli*.

Methylene-blue reduction test.—This test is now being used as a substitute for the enumeration of viable bacteria as described above. It depends on the decolorisation of the dye by the bacteria present in the milk, and the degree of bacterial contamination can be gauged by the time in which reduction and decolorisation takes place after methylene-blue has been added to a milk sample (*vide supra*). The procedure may be stated briefly as follows: 10 c.c. of the milk sample are placed in a special sterile test tube and 1 c.c. of a standard methylene-blue solution is added. The tube is closed with a sterile rubber stopper and the contents are carefully mixed. The tube is then placed at 37° C. in a water bath and observations are made every half-hour. Decolorisation is taken as complete when the milk is decolorised to within 5 mm of the top of the column.

For the detection of tubercle bacilli, a large quantity of the milk, *e.g.* 100 c.c., is thoroughly centrifuged, and from the sediment guinea-pigs are inoculated subcutaneously. The sediment may be examined microscopically for tubercle bacilli by the usual method (*vide* p. 120), but reliance can hardly be placed on the mere finding of acid-fast bacilli, and the animal test must be employed. The occurrence of tuberculosis in the inoculated animals constitutes conclusive evidence (*vide* p. 443). The lesions should be examined for tubercle bacilli to exclude pseudo-tubercle and other conditions that may simulate tuberculosis; it is noteworthy that *B. abortus* can produce a pathological condition in guinea-pigs resembling tuberculosis (*vide* p. 622). Direct cultivation (p. 444) may also be used for demonstrating tubercle bacilli in milk. In the detection of these organisms in milk some workers advise the examination of a mixture of centrifuged sediment and the separated cream.

Other pathogenic organisms that may occur in milk, e.g. *B. typhosus*, etc., can be demonstrated by the methods of cultivation appropriate to the particular organism, the sediment of a centrifuged specimen of the milk being used as the inoculum.

While these are the more important methods applicable to the bacteriological examination of milk in hygiene work, various other procedures have been employed in milk bacteriology; for these, reference should be made to works on agricultural and dairy bacteriology.

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[1] Under the Milk (Special Designations) Orders, 1936, for England and Scotland standard methods are described in official memoranda. Reference should be made to these for full details of such methods.

Antiseptics

The death of bacteria is judged of by the fact that, when they are placed on a suitable food medium, no development takes place; but, although not killed, bacteria under certain conditions may be deprived of their capacity to proliferate and to produce changes in organic matter. Owing to the importance of being able to kill or inhibit bacteria, an enormous amount of investigation has been directed to the means of doing so by chemical agencies. A substance having such a capacity is called an antiseptic, and the term is usually applied to substances which act in relatively high dilutions. Most antiseptics are general protoplasm poisons harmful to all forms of life, higher as well as lower, so that their use is limited to the inhibiting or killing of bacteria outside the animal body; still even this is of high importance. But all powerful antiseptics do not coagulate proteins such as serum or egg albumen.

Methods.—These vary very much. In early inquiries the amount of an antiseptic necessary to prevent putrefaction, *e.g.* in broth, urine, etc., was studied; but as bacteria vary in their powers of resistance, the method was unsatisfactory. It is now usual to estimate the effect of an antiseptic on pure cultures of pathogenic microbes, and in the case of a sporing bacterium, the effect on both the vegetative and spore forms is investigated. The organisms most used are the *Staphylococcus aureus*, *Streptococcus hæmolyticus*, *B. coli*, and the typhoid, cholera, diphtheria, and anthrax organisms—the last being employed for testing the action on spores. A good method is to wash off the growth from a twenty-four hours' agar-slope culture, and suspend it in 5 c.c. of sterile distilled water, remove coarse particles by centrifuging for two minutes and rejecting the sediment, add a measured quantity of this fluid to a given quantity of varying dilutions of the antiseptic dissolved in distilled water, then after the lapse of the period of observation, *e.g.* fifteen or thirty minutes, to remove one or two loopfuls of the mixture and place them in a great excess of culture medium; here it is preferable to use melted agar, which is then plated and incubated, since the number of colonies developing in comparison with the control, without antiseptic, shows the occurrence of bactericidal action even when all the organisms have not been killed. But fluid medium is very commonly used. In dealing with strong solutions of chemical agents it is necessary to be sure that the culture fluid is in great excess, so that the small amount of the antiseptic which is transferred with the bacteria may be diluted far beyond the strength at which it may be capable of inhibiting growth of the organisms. Sometimes it is possible at the end of the period of action to change the antiseptic into inert bodies by the addition of some other substance, but there is an objection to this procedure if a precipitate results, since the bacteria may be carried down with the precipitate and may escape the culture test. To test the effects of antiseptics on spores, Koch soaked silk threads in an emulsion

of anthrax spores and dried them. These were then subjected to the action of the antiseptic, well washed in water, and laid on the surface of agar. In using this method to test the efficiency of mercuric chloride it was found necessary to treat the organisms with ammonium sulphide, otherwise the antiseptic effect of traces of the mercuric chloride fixed by the spores went on after they were removed to the culture medium. As a rule, the method described, in which the small amount of antiseptic adhering to the bacteria is diluted with an excess of culture fluid, can safely be followed, especially when a series of antiseptics is being compared. Krönig and Paul introduced what is known as the "garnet method" for testing antiseptics. In this, small glass beads of equal size are carefully cleaned, dipped in a suspension of *B. anthracis* spores, and allowed to dry. They are then placed in the antiseptic solution, and from time to time some are removed, washed, and well shaken in a measured quantity of water. This is plated, and the number of colonies developing is counted.

In order to test a slowly acting antiseptic, varying concentrations of the latter are added to a constant volume of medium, *e.g.* 0·7 per cent. neutral peptone water, or sterile serum (previously heated at 56° C.), in a series of plugged sterile tubes; then the test amount of bacteria, *e.g.* a diluted young broth culture, is added to each; the tubes are incubated for twenty-four or forty-eight hours at 37° C. and the presence or absence of growth noted by the appearance of turbidity and by subculturing. Another method consists in adding varying amounts of the antiseptic to a series of tubes, each containing 10 c.c. agar medium. The mixtures are then poured into 3½-inch Petri plates. When the medium is set and its surface thoroughly dry, a stroke inoculation is made with a dilute suspension of a young culture of the organism to be tested. The dilution aimed at is such that on the control plate, without antiseptic, the stroke will yield a line of semi-confluent colonies. In this way a number of different organisms can be tested on the same plate. The results are read after forty-eight hours' incubation at 37° C.

The standardisation of antiseptics.—This has received much attention. A watery solution of carbolic acid is commonly taken as the standard with which other antiseptics are compared. The procedure in *Rideal and Walker's method* is to inoculate with a fixed amount (0·1 c.c. of bacterial suspension, prepared as described above, or of a twenty-four hours' broth culture) a fixed volume (5 c.c.) of a series of dilutions of the antiseptic to be tested, and at the same time a similar volume of a standard solution of chemically pure carbolic acid, *e.g.* 1 : 100; at short intervals (two and a half, five, seven and a half, and ten minutes) subcultures from all the tubes are made by inoculating 10 c.c. tubes of broth with a loopful (internal diameter of the loop = 4 mm.) of each of the mixtures, and these cultures are kept at 37° C. for forty-eight hours. The tubes containing the mixtures of antiseptic and bacteria are kept at a constant temperature (18° or 20° C.) in a water-bath during the period of the test. The results show (1) the time required to produce sterility with the concentration of carbolic acid used (by means of a preliminary test, the concentration of carbolic acid is determined which kills the test organisms in seven and a half minutes, but fails to kill them in five minutes; this is the concentration to be used), and (2) the highest dilution of the other antiseptic which

also produces sterility in the same time, say 1 : 3000. The latter substance is then stated to have a "carbolic acid coefficient" of 30, since under the conditions of the test the same bactericidal effect was produced by 1 : 100 carbolic acid. With a view to obtaining similar results in tests carried out on different occasions, it is usual to prepare the media under standard conditions as regards ingredients, *e.g.* Lab-Lemco and peptone, and pH ; also, always to use the same strains of organisms (*B. typhosus* or *B. coli*), and to subculture them at fixed intervals, *e.g.* to maintain the stock culture by subculturing once a month, and for four or five days before the test to subculture the organism daily.[1] It is to be noted that in comparing antiseptics by the above method the question of the organisms being killed at different rates by various substances is not taken into account (*vide infra*).

Chick and Martin's Method.—Since under practical conditions antiseptics are required to destroy organisms in solutions containing organic matter, the above method has been modified by causing the antiseptics to act on the bacteria in the presence of a suspension of 3 per cent. of dried sterilised human fæces. Cultures are made from the mixtures after thirty minutes' contact. Recently a suspension of commercial yeast has been recommended as an advantageous substitute for fæces (Garrod).

The Action of Antiseptics.—The action of antiseptics depends upon various factors. Thus the *medium* in which the bacteria are suspended is important ; most of the powerful antiseptics are greatly diminished in their action by the presence in the fluid of proteins or minute particles of organic matter which, according to Chick and Martin, act as adsorbing agents, and this is the reason why the action of such antiseptics on bacteria in fæces or sputum is limited in degree. The presence of *water* in the medium also plays an important part ; thus carbolic acid when dissolved in oil is a much less powerful antiseptic than when in watery solution ; and 70 per cent. alcohol kills organisms more rapidly than absolute alcohol. Again, the *temperature* at which the mixture of antiseptic and organisms is kept has a great influence : at 37° C. the lethal action occurs more rapidly and also takes place in higher dilutions than at lower temperatures. Other factors, such as the *hydrogen-ion concentration*, the degree of *ionisation*, and the dispersion of the antiseptic, may have a marked effect upon the lethal action of a given substance on bacteria. The first two of these factors are well illustrated by mercuric chloride, the antiseptic properties of which are due to the mercury ions ; increase in hydrogen-ion concentration brought about by adding dilute HCl increases the action, but the presence of NaCl, which reduces the ionisa-

[1] The specification of a standard method is obtainable from the British Standards Institution, 28 Victoria Street, London, S.W.1.

tion of the mercury salt, diminishes the antiseptic power. On the other hand, phenol, whose antiseptic effect is due to the molecule as a whole, is increased in its action by the presence of salt. In the case of mercuric nitrate it is stated that the antiseptic action is not diminished by the presence of NaCl. As regards the influence of *dispersion*, Chick and Martin found that certain antiseptics of the phenol group acted more powerfully when in the form of a fine emulsion than when in true solution, the former condition apparently favouring adsorption of the antiseptic by the bacteria.

The killing of bacteria by an antiseptic appears to proceed like a chemical reaction, such as the inversion of sugar by acid; thus Chick has shown that when the antiseptic is in excess, the number of organisms killed in a given time bears a fixed proportion to the number present at the beginning of the period of observation. Antiseptic properties, of course, depend essentially on chemical constitution. Some antiseptics, such as phenol or mercuric chloride, act rapidly, and concentrations which fail to kill the bacteria within a few hours may have little effect in inhibiting their multiplication subsequently. On the other hand, some basic organic dyes are only slowly lethal; at first they merely prevent proliferation of the organisms (bacteriostatic action), and twenty-four or forty-eight hours are required for the development of their maximum lethal effect. Another property of certain antiseptics is their selective action; for instance, with crystal violet Gram-positive organisms are in general more susceptible than Gram-negative; the concentration required to kill *B. coli* is five hundred times greater than that which kills staphylococcus. Both of these organisms, however, are practically equal in their susceptibility to mercuric chloride.

Nearly every substance which is not a food to the animal or vegetable body is more or less harmful to bacterial life. Thus neutral salts, *e.g.* NaCl, when present in high concentration act as preservatives by preventing the growth of bacteria, probably to a great extent through rendering unavailable the water which is essential for their proliferation. But, as has been stated, the term antiseptic is applied to substances which act in comparatively high dilutions. The most important antiseptics are the salts of the heavy metals, especially mercury and silver, certain acids (especially mineral acids), certain oxidising and reducing agents, volatile oils, and a great variety of organic chemical compounds. In comparing different antiseptics it is important to express their activity in terms of the molecular concentrations of the solutions used. When

this is done, certain important facts emerge. Thus the compounds of a metal of high atomic weight are generally more powerful antiseptics than those of one belonging to the same series, but of a lower atomic weight. Again, strong acids, *i.e.* those which are highly dissociated, *e.g.* HCl, are more powerfully antiseptic than the weak acids, *e.g.* boric, the H-ions in this case being responsible for the effect, although other factors may also be involved. With regard to oxidising agents and reducing agents, probably the possession of such properties has been overrated as increasing bactericidal potency. Thus in the case of such reducers as sulphurous acid and formic acid, the effect is apparently chiefly due to the fact that these substances are acids. Formic acid is much more efficient than formate of sodium. Hydrogen peroxide and permanganate of potassium are usually taken as the type of oxidising antiseptic agents; in the case of the latter, it can be shown that the greater amount of the oxidation which takes place when this agent is brought into contact with bacteria occurs after the organisms are killed. The essential chemical characters which determine the antiseptic properties of substances are, however, still obscure.

The Effects of certain Antiseptics.—Here we can only briefly indicate certain results obtained with the more common members of the group.

Halogens.—*Chlorine* dissolved in water is a very powerful antiseptic provided that practically no other organic material is present in addition to the bacteria. Hence it has been used on a large scale for the sterilisation of drinking-water supplies (1 to 2 parts of chlorine per million parts of water, thirty minutes being allowed for action). At the end of the action any excess of chlorine can be converted into harmless products by adding sodium thiosulphate. *Hypochlorites* (*e.g.* as Eusol) and *chloramine-T* (p-toluene sodium sulphochloramide) are similar in their action. A fresh 5 per cent. solution of chloramine-T added in the proportion of 2 volumes to 1 volume of tuberculous sputum will sterilise the latter in four hours (Uhlenhuth and Hailer). *Iodine* in the form of Liq. iodi mit. is used for sterilising the skin surface prior to surgical operations; but it may cause severe local irritation of the skin. In watery solution iodine is one of the most efficient agents for sterilising catgut for surgical sutures. The action of *iodoform* is obscure, as it shows no antiseptic action *in vitro*.

Mercury compounds.—The perchloride is a strong antiseptic, a 1 : 1000 solution being rapidly fatal to all non-spored organisms, and of all the salts of the heavy metals it has been most used. It is, however, a powerful poison for higher forms of life. Certain other salts of mercury, such as the oxycyanide, do not corrode metals as the perchloride does. Recently organic compounds of mercury have been introduced, *e.g.* mercurochrome (sodium salt of dibromoxy-

mercury - fluorescein), merthiolate (sodium ethyl mercurithio-salicylate), which, although powerfully antiseptic, are relatively non-toxic for mammalian tissues.

Phenol (*carbolic acid*), since its introduction for surgical purposes by Lister, has been widely used. As it is a much less powerful antiseptic than mercuric chloride, higher concentrations must be employed—usually a 1 : 20 or 1 : 40 solution. It is an active general protoplasm poison. Chemically analogous substances, the *cresols*, are stronger antiseptics than phenol; a mixture of these with soaps, *e.g.* liquor cresolis saponatus (B.P.) or lysol, has the advantage of detergent as well as antiseptic action and is used as a 5 per cent. dilution for treating infected articles. Krumwiede and Banzhaf recommend for the preservation of antiserum the addition of a mixture of equal parts of ether and cresol, so as to give a final concentration of 0·4–0·5 per cent. of cresol, with immediate shaking to cause a uniform mixture. Dettol is a mixture containing halogen derivatives of xylenols

Formaldehyde, used as formalin, a 40 per cent solution in water, is a powerful antiseptic and general poison It is employed for treating infected articles, and, on account of its being volatile, is used for room disinfection, *e g.* by spraying with 2 per cent. formalin. It tends to polymerise into inactive compounds.

Basic organic dye-stuffs.—Certain of these are very powerful bacteriostatic agents, and the extremely low concentrations which at first inhibit the activity of the bacteria, finally cause their death, *e.g.* brilliant green, proflavine (diaminoacridine hydrochloride), and acriflavine (diaminoacridine methochloride with an admixture of proflavine). Proflavine and acriflavine are not diminished in action by serum. They act best in an alkaline medium. On account of their low toxicity for mammalian tissues they are suitable for application to wounds, *e.g.* as a 1 : 1000 solution. An alcoholic solution of brilliant green and crystal violet (p. 163) is a powerful and unirritating antiseptic mixture for sterilising the skin surface before surgical operations.

Soaps have a marked bactericidal action on many pathogenic organisms; but staphylococci and the typhoid bacillus are resistant. Selective action is marked; *e.g.* pneumococcus, as compared with *Streptococcus hæmolyticus* is much more susceptible to oleate than to stearate or palmitate. Serum greatly diminishes this action of soaps (Walker). Other very actively antiseptic soaps have been investigated by Eggerth.

From the examples which have been given it will be recognised that the choice of an antiseptic and the precise manner in which it is to be employed depend on the conditions under which the bacteria are to be killed. In practice it is essential to use an antiseptic in sufficient concentration, to let it act for sufficient time, and to ensure that it penetrates the material to be disinfected, so as to come into contact with the bacteria. In illustration of the last point it may be mentioned that mercuric chloride solution will fail to disinfect sputum, because it coagulates the surface of the mass and so does not reach the interior.

In addition to chemical substances, certain *physical agencies* are harmful to bacteria. The importance of heat as a sterilising agent has already been dealt with. The action of heat depends greatly upon the presence of water, and organisms which are rapidly killed at 100° C. when in watery medium may when dry withstand prolonged heating at this temperature. This effect of water is no doubt related to its action in leading to the denaturation of proteins by heat. (The many practical applications of heat in pasteurising milk, sterilising canned foods, etc., cannot be dealt with in detail here.) On the other hand, very low temperatures, even that of liquid air, have little harmful effect on most bacteria. The bactericidal effect of radiations has been dealt with on p. 22.

Chemotherapeutic Agents

A chemotherapeutic agent is a substance of defined chemical constitution which conduces to cure of an infection by assisting the body in overcoming the causal organism. It appears that in most cases the drug or a derivative of it formed in the tissues, acts on the organisms by damaging their vitality, thus enabling the defensive mechanisms of the host to come into operation successfully. But it is possible also that a chemotherapeutic agent may produce its effect by stimulating these mechanisms. The oldest known chemotherapeutic agents are those which act in protozoal and spirochætal infections—quinine in malaria, mercury in syphilis, ipecacuanha in amœbic dysentery, and arsenious acid in trypanosomiasis. Only recently, from the stimulus of Ehrlich's work, has there been a systematic search for such drugs, the first synthetic compound being trypan-red, a single dose of which when given to mice cures a fatal infection with the trypanosomes of the disease of horses, "mal de caderas" (Ehrlich and Shiga). The first compound found to act on a general bacterial infection is optoquine, an analogue of quinine, with which Morgenroth cured pneumococcus infection in mice. A number of drugs have now been prepared which act on various infections. Difficulties attending such investigations on the chemical side are that the action of any given compound is usually restricted to a particular organism or to a small group of related organisms, and also that very few general relationships have so far been discovered between chemical constitution and therapeutic action. On the other hand, certain biological principles have been established. In the following account experimental results will be chiefly considered, practical applica-

tions being only touched upon. Trypanocidal agents will be dealt with first, since owing to their relatively large size and structural complexity trypanosomes lend themselves specially to detailed study. Then spirochæticidal agents will be treated, as spirochætes are very similar in their behaviour. Substances acting on malaria, and other protozoal and bacterial infections will be considered thereafter.

Methods.—The general procedure with any pathogenic agent is first to choose a host which shows a uniform behaviour when infected. Accordingly in the case of organisms which produce a general infection, the inoculum is selected of sufficient amount and virulence either to cause a fatal septicæmia in untreated animals or, where the infection tends to become latent or undergo cure spontaneously, to cause the parasites to appear in considerable numbers in the blood. In the case of local infections, either the effect on the lesion developing at the site of inoculation is studied or the influence on the general condition caused by the production and absorption of toxins. The toxicity of the drug having been ascertained in respect of the route of administration (subcutaneous, intravenous or intraperitoneal injection, or administration by mouth being usually employed) and the number and spacing of the doses, it is then administered to infected animals. At the same time a number of controls are left, similar in all respects but untreated. In this way a drug can be examined for curative action by beginning administration after inoculation or for prophylactic effect by reversing this order. Care must be taken not to miss therapeutic effects by giving the drug in too large doses, which may damage the animal's resistance and so conduce to infection, when smaller doses would be curative. When a substance has been shown to possess some therapeutic action the degree of efficacy can then be ascertained by such means as diminishing the dose, lengthening the interval between inoculation and treatment, or increasing the amount and virulence of the inoculum. A further important point is to examine the action of the drug on the infection in different species of hosts. It usually happens that in relation to their body weight small animals, as compared with larger ones, tolerate larger doses of the drug and that therefore a more effective therapeutic concentration is attained in their bodies. This is not an invariable rule, however, and various factors have to be considered—among others being the relative susceptibility of different organs to the drug in different species and also the varying course of infection in one animal as compared with another. The action of the drug on the organism *in vitro* should be examined also ; this test is most likely to give useful information in the case of substances which can be brought into close contact with infective organisms in a limited area, *e.g.* antiseptics for infected wounds. Even here the action of the tissues and the serum proteins in fixing the drug and so diverting it from the organisms, has to be reckoned with. Where a drug is required to reach the organisms by way of the blood or lymph streams, it may fail in the body though active *in vitro*. It may be toxic for some vital organ of the host or the tissues of the latter may absorb it and so leave too little

free to act on the parasites; or again it may be too rapidly excreted or destroyed. Of course, where a drug is excreted through a particular channel, *e.g.* by the kidneys, it is possible to concentrate its action on the organs of the excretory system concerned. On the other hand, substances with relatively little action on the parasite in the test-tube may be efficient chemotherapeutic agents.

Trypanocidal Agents.—There are now drugs belonging to a considerable number of chemical types which can cure trypanosomiasis, especially in small animals experimentally inoculated — pentavalent organic arsenicals (atoxyl, tryparsamide), trivalent organic arsenicals (arsenophenylglycine), bisazo dyes (trypan-red, trypan-blue), Bayer-205 (related to the previous group but not a dye), the diaminoacridinium dye trypaflavin (acriflavine), basic styryl quinoline dyes (Cohen's styryl-314), triphenylmethane dyes (parafuchsin, tryparosan). Specific action is very marked among trypanocidal agents; for instance, Bayer-205 and styryl-314 in a small fraction of the tolerated dose both cure mice infected with *T. brucei.* But in order to cure *T. congolense* infection in these animals a very high dose of the former is required, while the latter fails altogether. On the other hand, 7-amino 9*p*-aminophenyl 10-methylphenanthridinium chloride (synthesised by Morgan and Walls), which cures *T. brucei* only in the highest tolerated doses, is also curative for *T. congolense.* Drugs of two different types when administered within a short interval, may intensify each other's effect (potentation); or the one substance may abolish the therapeutic action of the other (chemotherapeutic interference phenomenon of Browning and Gulbransen). When a non-curative dose of a trypanocidal drug is given, the parasites may disappear from the blood for a time, but a relapse occurs later. The relapse may again be susceptible to treatment with the same drug but another relapse follows. After repeated relapses have been treated in this way, there may be finally no response to the largest dose of the drug. When the parasites are now inoculated into a fresh animal the infection which develops in it may also fail to be influenced by the drug. In fact a *drug-fast* strain has developed. When such drug-fastness has reached a high degree it may persist unaltered when the infection is passed for long periods through untreated animals. A chief factor contributing to this condition of the trypanosomes is that when brought into contact with the therapeutic agent they fail to absorb it; this can be strikingly shown by means of a coloured chemotherapeutic agent. Trypanosomes which have been rendered resistant to one class of drugs, *e.g.* the organic

arsenicals, remain normal in their susceptibility to other classes. On the other hand, when a strain has been rendered resistant to trypaflavin it has also become resistant to most other chemotherapeutic agents. An interesting morphological change in the trypanosomes accompanies the development of resistance to certain drugs chemically defined as of the ortho-quinonoid type of dyes, *e.g.* trypaflavin, namely, permanent loss of the blepharoplast. Of course, another form of insusceptibility to drugs may exist in which the parasites while absorbing the substance are insusceptible to its action. The destruction of the trypanosomes under the influence of a drug causes antibodies to be developed by the host. This immunity reaction may assist in bringing about cure after treatment. When a relapse occurs, however, the trypanosomes are found to have developed resistance to the antibodies present in the blood (p. 837). As regards the mode of action of trypanocidal drugs, it has been noted that many of the most effective have little effect on the trypanosomes *in vitro*, *e.g.* pentavalent organic arsenicals. Investigation of this phenomenon has shown that the related trivalent reduction product is powerfully trypanocidal. Therefore it has been concluded that there occurs in the body a gradual change of the drug from the inactive to the active form. Infected rabbits are most useful for the investigation of trypanocidal agents, since in them the disease has a subacute course similar to that of trypanosomiasis in larger domestic animals.

Bayer-205 has proved valuable in the treatment of infections with *T. brucei* and related trypanosomes in domestic animals, in camel trypanosomiasis and also in *T. rhodesiense* infection in man. Owing to its slow excretion it has a prolonged prophylactic action. Tryparsamide has been used with success in sleeping sickness in man; it is very little toxic and it penetrates into the cerebro-spinal fluid. Nagana has been cured in horses by styryl-314. For *T. congolense* infection in cattle antimony compounds are the most effective drugs available. *T. cruzi* is not susceptible to any drug so far known.

Spirochæticidal Agents.—The antisyphilitic property of mercurials has been long known, but is relatively feeble; recently bismuth has largely replaced mercury as an antisyphilitic. Ehrlich and Hata, proceeding on the analogy of the action on *Sp. minus*, the non-pathogenic parasite of mice, discovered the powerful therapeutic property of the trivalent arsenical, salvarsan, in experimental *Tr. pallidum* infection in rabbits, and subsequently its use in man has revolutionised the treatment of syphilis. It has also proved valuable in yaws, in infections

with *Sp. recurrentis*—although certain strains are resistant—and in rat-bite fever. The latter infection affords a striking example of one which is more susceptible to the therapeutic agent in man than in small animals. Leptospira infections are influenced by bismuth. The spirochætes, as compared with trypanosomes, show little tendency to acquire drug resistance. All efficient spirochæticidal agents so far are compounds of metals or metalloids, but it has been shown that purely organic substances may have some therapeutic effect on *Sp. minus* (Browning and Gulbransen).

Anti-Malarial Drugs.—In addition to quinine and certain of its analogues, as well as methylene-blue and salvarsan, new anti-malarials have been discovered as a result of experimental investigations on analogous infections in birds. Atebrin, an acridine derivative, acts like quinine on the parasites in phases of their asexual cycle. Plasmoquin, a quinoline derivative, on the other hand, acts on the crescents, and so by destroying the gametocytes prevents transmission of the infection to the mosquito. Atebrin and plasmoquin both have a marked prophylactic action when administered shortly before and for a few days after the bite of the infected mosquito; but quinine lacks this action. In the case of *P. vivax*, if complete sterilisation is not effected an attack of malaria develops after a long latent period, *e.g.* of eight months (James). The mechanism of the spontaneous remission in malarial infections is obscure and likewise the therapeutic effect of drugs.

Chemotherapeutic Action on Other Protozoa.—Antimonials act in leishmaniasis. Emetine is effective in amœbic dysentery apparently on account of its direct lethal action on *E. histolytica*, which is also manifested in cultures. In piroplasmosis trypan-blue, trypaflavin, and especially acaprin (an acridine derivative) act well on infections with the larger forms of this group (babesia). Acaprin alone influences infections with certain of the minute theileria types.

Antibacterial Agents.—In local infections where the drug can be brought into close contact with the organisms there is a field for the application of antiseptics which possess a high degree of inhibitory action on the bacteria while being relatively non-toxic to mammalian tissues. Such properties characterise especially proflavine and acriflavine. The efficacy of these compounds over phenol, for example, is well brought out in experimental wound infections of guinea-pigs with *B. diphtheriæ*. A brief treatment of the infected wounds with a solution of acriflavine prevents development of the organisms and death

of the animals from the characteristic intoxication, whereas washing of the wounds with physiological or hypertonic saline is ineffective and phenol has only a slight action. Also, after intraperitoneal inoculation of mice with virulent streptococci the injection into the peritoneum of acriflavine or certain anil quinoline compounds is highly effective in saving the life of the animals. From estimations of the bactericidal action of these drugs on the organism *in vitro*, it is clear that the therapeutic effect is not due to rapid sterilisation of the peritoneal contents, but that the substances act by reducing the virulence of the organisms and so allow the tissue defences to co-operate in bringing about cure. In connection with the treatment of local infections it should be remembered that drugs cannot penetrate masses of necrotic tissue and therefore that the chemotherapy of wound infections must be accessory to, and not a substitute for, operative measures. The high prophylactic value of these drugs finds a valuable application where operations must be undertaken in areas which are already infected, *e.g.* mucous membranes. Bacteria do not readily become resistant to these agents.

The sanocrysin (gold sodium thiosulphate) treatment of tuberculosis and Walbum's metallic salt therapy of various infections appear to depend on a stimulation of the defence mechanisms rather than on antiseptic action, but the results lack general confirmation. The treatment of a general bacterial infection, as already mentioned, was first realised by Morgenroth with optoquine in mice infected with pneumococci. The action of this drug is due apparently to its powerful bactericidal effect which is specific for pneumococci. Apart from the treatment of pneumococcal ulcer of the cornea, it has not been much used in man on account of its toxic effects. Recently an azo dye, prontosil, and more particularly a colourless moiety, *p*-amino benzene sulphonamide, have been shown to possess marked action on general streptococcal infections in mice, the life of the animals being saved after intraperitoneal inoculation when soon afterwards the drug is injected subcutaneously and treatment is repeated several times. Results in rabbits have also been successful. Infections with other organisms in mice have been favourably influenced, however. The drug has been used in the human subject in puerperal streptococcal infection and in erysipelas, and favourable results have been recorded. As these compounds are not highly bactericidal *in vitro*, it appears that they do not act merely as internal antiseptics.

APPENDIX

THE BACTERIAL FLORA OF THE NORMAL SKIN AND MUCOUS MEMBRANES

THE skin and mucous membranes, in virtue of being in more or less direct contact with the outer world, are liable to contamination with various micro-organisms. In addition, the local chemical and physical conditions at a surface may determine what organisms will multiply there; also, the normal flora may influence the growth of any superadded bacteria. As will be seen, the commensal flora in different parts of the body may be very diverse. It is obvious that when considering what constitutes an abnormal flora, which may have pathogenic significance, it is necessary to know the normal condition both in its qualitative and quantitative aspects. Accordingly, the organisms should be studied both in direct films and also in cultures on various media. Types which are abundant may fail to grow on ordinary media, *e.g.* spirochætes, or as in the case of *B. bifidus*, the predominating organism in the fæces of breast-fed infants, may require anaerobic conditions for their initial growth. The use of selective enrichment media may be necessary in order to demonstrate the presence of organisms which are either extremely scanty, such as *B. aerogenes* in normal fæces, or which are readily overgrown, *e.g. B. typhosus* when along with the usual coliform bacilli. Pathogenic forms may be transiently present without disease resulting. The conditions which determine the normal flora of a part are as yet only imperfectly understood. Accordingly one can only note the fact that in different parts of the world the organisms found, say in the upper respiratory tract, may vary.

The Skin.—Except at flexures, the skin is too dry to permit free growth of bacteria; also, a self-cleansing action has been attributed to it whereby bacteria placed on the surface soon disappear. But certain organisms are habitually found, such as staphylococci, *M. tetragenus,* diphtheroids and various forms which are present in the air and dust. Staphylococci and the acne bacillus appear to inhabit the deeper layers of the skin,

so that complete sterilisation cannot be effected by the application of antiseptics to the surface. It is of interest that pyogenic staphylococci are rare or absent from the fingers of persons who are free from pyodermia, but are usually present when infection of any part of the skin exists. Whether this is due to contamination from the infected site or is an indication that the skin in general is specially susceptible to infection in these cases, is not clear. Where surface contamination is abundant, as at the muco-cutaneous junctions, the organisms from the mucous membrane are numerous, *e.g.* coliform bacilli and enterococci near the anus. The preputial secretions favour the growth of smegma bacilli, Gram-negative and Gram-positive cocci, diphtheroids, fusiform bacilli and various spirochætes. The external genitalia of the female have a similar flora. On the conjunctiva, *Staphylococcus*, especially *albus*, and *B. xerosis* are found. Where there are fatty or waxy secretions acid-fast organisms, smegma bacilli, tend to flourish.

The Mouth.—In the mouth a great variety of organisms is found and any accumulation of secretions or epithelial debris leads to increase in their number. Streptococci of α and γ types, spirilla and vibrios, leptothrix, and streptothrix forms and also potentially pathogenic types such as pneumococci, staphylococci and fusiform bacilli are all frequent. In the interstices between the teeth and on the gums, abundant spirochætes of various kinds are often present. Where there are carious teeth, *B. acidophilus odontolyticus* is present, but it is uncertain in how far this is the cause of caries.

The Respiratory Tract.—While the nasal vestibule contains many and varied organisms, *Staphylococcus albus* and *aureus*, diphtheroids, etc., the nasal fossæ, so far as they are accessible, are practically sterile. Even slightly abnormal conditions, however, such as those associated with the common cold, lead to the appearance of organisms, which may be numerous, such as staphylococci, streptococci, Friedländer's bacillus, *M. catarrhalis* and diphtheroids. These probably prolong the effects of the original infection, although they may not initiate it. The nasal mucous membrane has a marked capacity for ridding itself of bacteria placed on it; the mechanism involved is not clear, however, although it seems not to depend entirely on mechanical removal. The *nasopharynx* harbours non-hæmolytic streptococci, non-pathogenic Gram-negative cocci, pneumococci (chiefly of Group IV), staphylococci, bacilli of the influenza group and a filter-passer allied to *B. pneumosintes*. The *paranasal sinuses* are normally

sterile. Friedländer's bacillus is a common inhabitant of the upper respiratory tract and occurs also in the mouth. The *trachea, bronchi,* and *pulmonary tissues* in the healthy state are practically free from organisms.

The Alimentary Tract.—In the adult, vegetative forms of the bacteria swallowed with the food and saliva are largely destroyed by the hydrochloric acid of the gastric juice, so that when the stomach has emptied after a meal its contents are nearly sterile. The duodenum also contains very few organisms, which are of the intestinal types. As distance from the pylorus increases after the first 2–6 feet the numbers of organisms increase until they are abundant in the ileum and reach their maximum in the cæcum and colon. Lower down the large intestine, inspissation and other factors lead to the death of a considerable proportion. It has been estimated that nearly one-third of the dry weight of the fæces consists of bacteria. The relative proportions of the different types depend to a considerable extent on the nature of the diet, but the principal forms are coliform bacilli, *B. proteus,* enterococci, aciduric bacilli (*B. acidophilus*), *B. pyocyaneus,* anaerobic bacilli (*B. welchii* and *B. sporogenes,* etc), aerobic sporing bacilli (*B. subtilis, B. mesentericus*) and, in addition, there are spirochætes, moulds and yeasts. The organisms present in the intestines may be classified according as they are saccharolytic (enterococcus, *B. acidophilus, B. welchii*) or proteolytic, *i.e.* "putrefactive" (*B. sporogenes, B. proteus, B. pyocyaneus, B. mesentericus*). Diet is believed to influence the flora, a large proportion of meat conducing to the proteolytic types, whereas carbohydrates—especially lactose which is supposed to reach the lower parts of the intestine before being split up—lead to the preponderance of an aciduric flora. Feeding with soured milk assists in establishing the latter. It is not easy, however, to cause an organism foreign to the individual's intestine to flourish there, although abnormal conditions, such as enteritis, frequently lead to an altered flora, *e.g.* in bacillary dysentery after the specific bacteria have disappeared. The maintenance of the normal flora may depend in part on the antagonism of the organisms present to others, *e.g.* strains of coliform bacilli tend to suppress *B. typhosus* in cultures. It is doubtful whether bacteriophage plays much part in regulating the intestinal flora. For instance, in the case of a chronic carrier of *B. paratyphosus B,* a phage for the patient's own strain has been recovered from the fæces. Bacteria which have been swallowed appear in the dejecta of new-born infants within twenty-four hours; at first they are of various kinds but

when breast-fed, *B. bifidus* soon becomes the predominating and almost the sole organism and this condition is maintained till weaning. In artificially fed infants there is a mixed flora in which coliform bacilli are prominent. Blacklock and others have found that in such infants where the alimentary tract is normal, the upper part of the small intestine is nearly sterile, and that this is not due to the acidity of the contents or to the presence of bacteriophage.

The Female Genital Tract.—In the adult female the vaginal secretion is typically acid from lactic acid derived from glycogen secreted in the lining epithelium. This state persists from the onset of puberty until the menopause. The acid secretion is unfavourable for the majority of organisms and the characteristic flora consists of the aciduric Döderlein's bacillus (*B. acidophilus*). In a proportion of pregnant women, however, the flora consists to a greater or less extent of other organisms such as streptococci, coliform bacilli, diphtheroids, yeasts and sarcinæ. In children from three to four weeks old until puberty, the vaginal secretion is alkaline and contains few organisms, chiefly cocci.

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CHAPTER I.—General Morphology and Physiology

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CHAPTER II.—Methods of Cultivation of Bacteria

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INDEX

Abortus fever, 622.
Abrin, 196, 208, 218.
Abscesses, 285.
  in amœbic dysentery, 818, 819.
  pyæmic, 313.
Absorption method for agglutinins, 141.
Acaprin as chemotherapeutic agent, 918.
Achoria, 867.
Achorion quinckeanum, 869.
  schönleinii, 869.
Acid-fast bacilli, non-pathogenic, 428.
    stain for, 120.
Acidophile bacteria, 900.
Acids as antiseptics, 912.
Aciduric bacteria, 900.
Acne, 313.
Acquired immunity, 200.
Acriflavine as antiseptic, 913.
  as chemotherapeutic agent, 918.
Actinobacillus, 37, 466, 470.
  lignièresi, 477.
  mallei, 456.
  pseudomallei, 464.
Actinomyces, 37, 361, 466.
  actinoides, 477.
  asteroides, 477.
  bovis, 473.
  characters of, 466.
  clubs in, 469.
  conidia of, 468.
  cultivation of, 473, 481.
  farcinicus, 478.
  graminis (Boström), 475.
  gypsoideus, 478.
  maduræ, 479.
  necrophorus, 478.
  spores of, 468.
Actinomycetaceæ, 36.
Actinomycetales, 36.
Actinomycosis bovis, 473.
  diagnosis of, 481.
  lesions in, 470.
  source of infection, 472.
Active anaphylaxis, 262.
  immunity, 201, 202.
Acute disseminated encephalomyelitis, 761.
  rheumatism, 775.
Adhesion phenomenon of Rieckenberg, 237, 837.
Aëdes ægypti, 779, 784.
  calopus, 779.
Aerobacter, 35, 514.
Aerobes, 17.
Aerobic organisms, separation of, 88.
“ Aestivo-autumnal ” fevers, 802.
African horse sickness, 721.
  tick fever, 690.
Agalactia, organisms of, 484.
Agar media, 60.
Agglutinable cultures, standard, 140.
Agglutinating sera, method of obtaining, 142.
Agglutination, 232.
  absorption, Castellani’s reaction, 236.
  absorption method, 141.
  and complement fixation, 243.
  differentiation of bacteria by, 235.
  flagellar, 234.
  group, 236.
  H, 234.
  in Malta fever, 619.
  mechanism of, 239.
  methods, 138.
  nature of, 236, 239.
  non-specific stimulation of, 236.
  O, 234.
  somatic, 141, 234.
  in typhus fever, 709.
  Vi, 142.
Agglutinins (*see* Agglutination), 232.
  primary and secondary, 542.
  varieties of, 233, 234.
Agglutinogens, 234.
  non-protein, 241.
Aggressins, 192, 231.
Air, bacteriology of, 885.
  methods of examination of, 885.

Alastrim, 733.
Alcaligenes, 36.
  fæcalis, 554.
Alcohol as antiseptic, 910.
Aleuronat, intrapleural injection of, 281.
Alexin, 224, 254.
Alimentary tract, normal bacterial flora of, 922.
Allergy, 176, 193, 260, 268, 302.
Amboceptor, 224, 226.
Amœba coli, 810.
Amœbæ, intestinal, non-pathogenic, 814.
Amœbic dysentery, 809.
  carriers of, 813.
  chemotherapy of, 918.
  lesions in, 818, 819.
  methods of examination in, 821.
Anaerobes, 625.
  cultures of, 97.
  Fortner's method for, 96.
  Henry's method for, 95.
  in infected wounds, 645.
  proteolytic, 647.
  saccharolytic, 647.
  separation of, 97.
Anaerobic bacilli, 625.
  fusiform, 662.
  bacteria, 17.
  organisms, culture of, 92.
  streptococci, 291, 292, 295, 305.
Anaphylactin, 265.
Anaphylactogens, 260.
Anaphylatoxin, 266.
Anaphylaxis, 193, 260 *et seq.*
  active, 262.
  complement in, 267.
  mechanism of, 265.
  passive, 263.
  post-mortem changes in, 264.
  and precipitins, 265.
  sensitisation in, 262.
  symptoms in, 264.
  theories of, 265.
Anaplasma, 857.
Anatoxin, 221.
Andrade's indicator, 79.
Anil quinoline compounds as chemotherapeutic agents, 919.
Aniline dyes, 113.
Aniline-xylol, 112.
Animal poisons, 196.
  immunity against, 218.
Animals, autopsies on, 164.
  inoculation of, 160.
Anopheles and malaria, 804.
Anthrax, 486.
  in animals, 493.
  antibody reactions, 503.
Anthrax, Ascoli's thermo-precipitin reaction in, 503.
  diagnosis of, 504.
  disposal of animal carcases, 500.
  in human subject, 495.
  immunity against, 500.
    mechanism of, 502.
  intestinal, 499.
  Pasteur's method of vaccination against, 500.
  protective inoculation, 501.
  spread of, 499.
Anthrax bacillus, biology of, 490.
    capsulation of, 491.
    characters of, 486.
    cultivation of, 488.
    experimental inoculation with, 495.
    McFadyean's reaction for, 487.
    organisms related to, 505.
    in soil, 890.
    sporulation of, 490.
    variants, 492.
Anti-abrin, 218.
Antiagressins, 241.
Anti-anaphylaxis, 263.
Anti-anthrax sera, 502.
Antibacterial serum, 222.
  properties of, 223.
Antibodies, 209.
  action of, 211.
  chemical nature of, 245.
  Forssman's, 228.
  heterophile, 228.
  mechanism of formation, 246.
  in milk, 246.
  nature of, 243.
  non-specific formation of, 249.
  plurality of, 242.
  site of origin of, 244.
  source of, 243.
  specificity of, 210.
  unity of, 242.
  varieties of, 211.
  Vi, 234.
Anti-cholera inoculation, 575.
Anti-diphtheritic serum, 214.
Anti-dysenteric serum, 551.
Antiformin in examining for tubercle bacilli, 443.
Antigen-antibody complex, 246.
Antigens, 209.
  heterogenetic, 228.
  "mosaic," 236.
  Vi, 234.
  for Wassermann reaction, 154.
Anti-haemolysin, 282.
Anti-meningococcic serum, 358.
Antimonials as chemotherapeutic agents, 918.
Anti-plague sera, 609.

Anti-pneumococcal serum, 339, 340, 344.
Anti-ricin, 218.
Antiseptics, 908.
  Chick and Martin's method for, 910.
  mercuric chloride, 909.
  methods of testing, 908.
  Rideal and Walker's method for, 909, 910.
  selective action of, 911.
  standardisation of, 909.
  varieties of, 912.
Antisera, therapeutic use of, 249.
Anti-streptococcal serum, 309.
Anti-tetanic serum, 637.
Antitoxic action, nature of, 218.
    theories of, 219.
  power, estimating of, 216.
  serum, 213.
Antitoxin, development of, 214.
  diphtheria, use of, 394.
  immunity unit of, 219.
  mechanism of formation, 247.
  standardisation of, 214, 216.
Anti-tuberculous sera, 442.
Antivenin, 218.
Apotoxin, 266.
Appendicitis, 298, 317.
Archimycetes, 860.
Arsenicals as chemotherapeutic agents, 916.
Arsenophenylglycine as chemotherapeutic agent, 916.
Arthrospores, 7.
Artificial immunity, 201.
    methods of, 202.
Asci, 859.
Ascocarp, 860.
Ascoli's thermo-precipitin reaction in anthrax, 503.
Ascomycetes, 859.
Ascospores, 860.
Aspergillosis, 876.
Aspergillus, 481.
  fumigatus, 876.
  herbariorum, 861.
  pictor, 876.
Atebrin as chemotherapeutic agent, 918.
Atopy, 269.
Atoxyl as chemotherapeutic agent, 916.
Attenuation of virulence, 203.
Australian encephalitis, 761.
Auto-agglutination of blood in trypanosomiasis, 838.
Autoclave, use of, 53.
Autolysis, 192.
Autopsies on animals, 164.
Avian tuberculosis, 426.
Axoneme, 824, 846.

Babesia bigemina, 855.
  bovis, 855.
  canis, 855.
  equi, 855.
Bacillaceæ, 36.
Bacillary dysentery, 546.
  necrosis, 478.
  white diarrhœa of chicks, 545.
Bacilli, acid-fast, non-pathogenic, 428.
    stain for, 120.
Bacillus, 2, 30, 36.
  abortus (*see* Brucella), 361, 620
    agglutination of, 622.
    bovine type, 621.
    characters and cultivation of, 620.
    equi, 543, 544.
    infection, diagnosis of, 623, 624.
    in milk, 622.
    ovis, 543, 544.
    porcine type, 621.
    in undulant fever, 622.
  acidophilus, 900, 901.
  acne, 313.
  actinomycetem comitans, 474.
  ærofetidus, 662.
  ærogenes, 514.
    encapsulatus, 647.
  ærtrycke, 534, 539, 544.
    infection of eggs, 540.
  alkalescens, 552.
  anthracis (*see* Anthrax bacillus), 361, 486.
  anthracoides, 505.
  avisepticus, 612.
  bifermentans, 662.
  bifidus, 900, 901.
  of Bordet and Gengou, 590, 591.
  botulinus, 641.
    characters of, 642.
    pathogenic action of, 643.
    types of, 642, 644.
  bovisepticus, 612.
  bronchisepticus, 624.
  chauvœi, 658.
  cloacæ, 514.
  cochlearius, 662.
  coli, 507.
    action on neutral red, 511.
    agglutination of, 512.
    anærogenes, 509, 515.
    atypical, 892.
    cellobiose fermentation, 515.
    characters of, 507.
    citrate-utilisation, 511, 515.
    $CO_2 : H_2$ ratio, 510.
    communior, 513.
    communis, 513.

Bacillus coli communis (Escherich), 507.
cultural reactions, 509.
hæmolysis, 512.
as indicator of recent fæcal contamination, 514.
infections, 317.
inosite-fermenters, 514.
isolation of, 512.
lesions caused by, 317.
measuring of gas formation, 510.
methyl-red reaction, 510.
in milk, 902, 907.
pathogenic properties of, 515.
production of indole, 511.
reactions of, 509.
reduction of nitrates, 511.
in soil, 889.
typical, 512.
variation and mutation, 515.
varieties of, 513.
Voges and Proskauer's reaction, 510.
in water, 891.
of Danysz, 538.
diphtheriæ (*see* Diphtheria bacillus), 373.
dispar, 552.
dysenteriæ (*see* Dysentery bacilli), 546.
enteritidis, 544.
var. Dublin, 544.
of Gærtner, 538.
sporogenes (*see* B. welchii), 647.
fæcalis alkaligenes, 549, 554.
blood infections, 555.
fallax, 654.
funduliformis, 479.
fusiformis, 662.
gallinarum, 544, 545.
granulosis, 788.
of guinea-pig pneumonia, 624.
hæmoglobinophilus canis, 585, 586.
IX of von Hibler, 660.
histolyticus, 661.
of Hofmann, 401.
influenzæ (*see* Influenza bacillus), 361, 579.
suis, 585, 771, 772.
of Johne's disease, 446.
Koch-Weeks, 314, 586.
lactis aerogenes, 514.
lepisepticus, 612.
of leprosy, 447.
of malignant œdema, 654.
mallei, 456.
megatherium, 505.
of melioidosis, 464.
Bacillus melitensis (*see* Brucella), 615.
agglutination reaction, 619.
characters of, 616.
cultivation of, 616.
in milk, 903.
relations to disease, 617.
mesentericus (vulgatus), 505.
"Mist" (Mœller), 428.
Morax-Axenfeld, 315.
morbificans bovis, 540, 544, 545.
of Morgan No. 1, 554, 549.
of Muller, 315.
multifermentans tenalbus, 662.
mycoides, 505.
necrophorus, 478.
novyi, 659.
œdematiens, 659.
ozænæ, 321, 350.
para-abortus, 623.
para-botulinus, 644.
paracolon, 549, 554.
para-melitensis, 623.
para-sporogenes, 662.
paratyphosus A, 534, 543, 544.
B, 534, 543, 544.
d-tartrate utilisation, 541.
Pesch's test, 541.
schleimwallbildung, 535.
C, 534, 543, 544.
perfringens, 647.
pertussis, 590.
characters of, 591.
cultivation of, 592.
differentiation from B. influenzæ, 595.
methods of examination, 595.
pathogenic effects of, 593.
serological reactions, 592.
vaccine, 594.
pestis, 596, 597.
antigenic structure, 609.
biological reactions, 599.
cultivation of, 598.
distribution of, 600.
experimental inoculation with, 602.
immunity in, 607.
infection, part played by rat fleas, 603.
paths and modes of, 603.
of rats, 603.
involution forms on salt agar, 598.
pathological effects of, 600.
stalactite growth, 599.
toxins, 607.
viability of, 599.
phlegmonis emphysematosæ, 647.
proteus, 320.
Kingsbury, 321.

Bacillus proteus, O-XK, 711.
X 19, 321, 709.
pseudo-diphtheria, 401.
pseudo-tuberculosis ovis, 402.
rodentium, 612.
pullorum, 544, 545.
putrificus, 661.
pyocyaneus, 318.
pyogenes, 403.
of Rauschbrand, 658.
of rhinoscleroma, 349.
rhusiopathiæ, 482.
of Schmitz, 551.
simiæ, 788.
smegmatis, 429.
of soft sore, 321.
sphenoides, 662.
sporogenes, 660.
subtilis, 505.
suipestifer, 539, 543, 544.
(monophasic), 544.
suisepticus, 612.
tertius, 660.
tetani (*see* Tetanus bacillus), 625.
tatanomorphus, 662.
Timothy grass, 428.
of tuberculosis (*see* Tubercle bacillus), 361, 405.
typhosus (*see* Typhoid bacillus), 361, 517, 544.
welchii, 647.
antitoxin, 651.
cultural characters of, 648.
experimental inoculation, 651.
pathogenic effects of, 649.
in soil, 889.
toxins of, 651, 653.
types pathogenic for sheep, 653.
variants of, 652.
in water, 893.
whitmori, 464.
of whooping-cough, 590, 591.
xerosis, 316, 402.
Y of Fleming, 660.
Bacteria, action of, 23.
American classification of, 32.
anaerobic, 17.
associated with inflammatory and suppurative conditions, 274.
autotrophic, 14.
capsulated, 4.
chemical composition of, 12.
classification of, 27.
counting of, 159.
differentiation of by agglutination, 235.
effect of light on, 22.
moisture on, 17.
electric charge of, 14.
Bacteria, ferments of, 26.
food supply of, 14.
gaseous environment, relation to, 17.
heterotrophic, 15.
higher, 3, 31.
reproduction among, 31.
lesions produced by, 183.
lower, 2, 27.
reproduction among, 5.
metabolism of, 14.
methods of counting, 159.
micro-aerophilic, 18.
microscopic examination of, 107.
modes of division of, 6.
morphology of, 4.
motility of, 10, 22.
nitrifying, 24.
nitrogen-fixing, 24.
physical characters of, 13.
physiology of, 14.
purple, 22.
pyogenic, 276, 323.
relations of, 3.
to oxygen, 18.
to temperature, 21.
reproduction of, 5.
respiratory mechanisms of, 18.
in sections, 111.
staining of, 112.
structure of, 4.
sulphur-containing, 32.
temperature relations of, 21.
thermophilic, 21.
variation among, 38.
vitamins in, 13.
water requirements of, 17.
Bacteriaceæ, 35.
Bacteriæmia, 181, 275.
Bacterial action, effects of, 182.
methods of, 25.
pathogenic, modes of, 180.
cultures, mounting of, 103.
ferments, 26.
flora, normal, of skin, etc., 920.
infections, chemotherapy of, 918.
pigments, 13.
protoplasm, minuter structure of, 10.
toxins, 189.
Bactericidal action, 223.
of serum, 145.
of whole-blood, 147.
effect of radiations, 914.
methods, 145.
powers, natural, 252.
Bacteriolytic action, 223.
Bacteriophage, 41.
method of obtaining, 43.
nature of, 45.
plague, 610.

Bacteriophage, resistance of, 44.
therapy in cholera, 573.
in dysentery, 553.
Bacteriostatic action, 911.
Bacteriotropin, 229.
Bacterium anatum, 544, 546.
monocytogenes, 397, 403.
pneumosintes, 588.
tularense, 613.
Bacteroides, 35.
Bartonella bacilliformis, 856.
infections, 856.
muris, 857.
Basidia, 859.
Basidiomycetes, 859.
Bayer-205 as chemotherapeutic agent, 916.
Becker's method (modified) for staining spirochætes, 128.
Beer wort agar, 76.
Beggiatoa, 32.
"Behring venule," 167.
Berkefeld filter, 99.
Bezel, 677, 681.
Bile-salt lactose agar, MacConkey's, 73.
Bismarck-brown, 113.
Black-quarter, 658.
"Black tongue," 884.
Blackwater fever, 808.
Blastomycosis, 880, 881.
examination of, 882.
generalised, 882.
Blastomycotic dermatitis, 881.
Blepharitis, 285.
Blepharoplast, 824, 917.
Blood agar, 69.
cultures, 165.
films for malaria diagnosis, 809.
media, 68.
method of obtaining, 165.
samples from rabbits, 149.
B-lysins, 255.
Boas-Oppler bacillus, 901.
Boeck and Drbohlav's medium, 817.
Boiled blood medium, 69.
Boils, 285.
Boöphilus bovis, 855.
Bordet and Gengou, bacillus of, 590, 591.
medium, 69.
reaction of, 151.
Borna disease, 762.
Borrelia, 37, 666.
recurrentis, 682.
Boström's actinomyces, 475.
"Botryomycosis," 280.
Botulinus bacillus, 641.
Botulism, 641.
Bouillon, 55.
Bovine abortion, 622.
Bovine mastitis, 291, 292, 294.
Bovine tuberculosis, 421.
Braxy, 658.
Brilliant green as antiseptic, 913.
enrichment method, 74.
Bromcresol purple, 79.
Bronchitis, 298.
Broncho-moniliasis, 875.
Broncho-pneumonia, 298, 336, 342.
Brownian movement, 14.
Brucella, 36.
abortus, 620.
group, 615.
carbon dioxide requirements, 621.
differentiation of members, 621.
hydrogen sulphide formation, 621.
selective action of dyes, 621.
serological characters, 623.
melitensis, 615, 616.
suis, 621.
Bubonic plague, 600.
Buchner's tube, 95.

Calmette's BCG vaccine, 439.
tuberculin ophthalmo-reaction, 434.
Capillary pipette method of making dilutions, 137.
Capsule formation, 213.
Capsules, Hiss's method for staining 122.
J. Kirkpatrick's method for staining, 123.
Rd. Muir's method for staining, 123.
relief staining for, 124.
Carbol-fuchsin, dilute, 116.
Ziehl-Neelsen, 116.
Carbol-gentian-violet, 117.
Carbol-thionin, 116.
Carbolic acid as antiseptic, 913.
Carbuncles, 285.
Carriers, 176.
of amœbic dysentery, 813.
cholera, 566.
diphtheria, 389.
of meningococcus, 355.
typhoid, 527.
Carrot as culture medium, 77.
Casein digest broth, 58.
Castaneda's staining method, 725.
Castellani's reaction, 236.
Catalase, 20.
Catarrhal inflammations, 298.
Catarrhs of mucous surfaces, 285.
Cattle plague, 721.
Cell inclusions, 719.
Centrifuges, 102.
Ceratophyllus fasciatus, 605, 843.

Cerebro-spinal fever, 350.
  fluid, examination of, 167.
  Wassermann test of, 155.
  meningitis, 350.
Chagas' disease, 838.
Chamberland filter, 99.
Chancroid, 321.
Charbon (*see* Anthrax), 486.
  symptomatique, 658.
Chemical composition of bacteria, 12.
Chemotaxis, 23.
Chemotherapeutic agents, 914.
    methods of testing, 915.
  interference, 916.
  potentation, 916.
Chick and Martin's method for antiseptics, 910.
Chicken pox, 766.
Chlamydobacteria, 32.
Chlamydozoa, 720.
Chloramine-T as antiseptic, 912.
Chlorine as antiseptic, 912.
Chocolate agar, 69.
Cholecystitis, 297, 298, 313, 317.
Cholera, 560.
  bacteriophage, 572.
  carriers, 566.
  methods of diagnosis, 576.
  vaccination against, 575.
  vibrio, 560.
    agglutination of, 571.
    antigenic structure, 574.
    biological reactions of, 564.
    characters of, 560.
    cultivation of, 562.
    distribution of, 561.
    experimental inoculation with, 567.
    experiments on human subject, 568.
    hæmolytic test, 565.
    Heiberg's criteria, 574.
    immunity to, 570.
    infection with, 566.
    isolation from water, 896.
    organisms related to, 573.
    Pfeiffer's reaction, 570.
    powers of resistance of, 566.
    toxins of, 569.
    variants, 563.
    viability, 566.
    vibrios allied to, 574, 577.
Cholera-like vibrios, 574, 577.
Cholera-red reaction, 565.
Chorio-meningitis, lymphocytic, 762.
Chromidial bodies, 813.
  buds, 825.
Chrysoidin, 113.
Chytridiinæ, 860.
Cimex lectularius, 703.
  rotundatus, 849.
Citrate utilisation, 511.
Cladothrix, 32.
  asteroides, 477.
Clarification, 102.
Classification of bacteria, 32.
Climatic bubo, 787.
Clostridium, 36.
  agni, 653.
  botulinum, 641.
  chauvœi, 658.
  genus, 646.
  histolyticum, 661.
  œdematiens, 659.
  œdematis maligni, 654.
  ovitoxicum, 653.
  paludis, 653.
  sporogenes, 660.
  tertium, 660.
  tetani, 625.
  welchii, 647.
Clubs in actinomyces, 469.
Co-agglutination, 235.
Coagulase, 283.
Coccaceæ, 34.
Cocci, 2, 27.
  pyogenic, 274.
Coccidioides, 880.
  immitis, 880.
Coccobacillus fœtidus ozænæ of Perez, 321.
Cold, common, experimental transmission of, 773.
Coli-typhoid group, 506.
    biological reactions, table of, 556.
    isolation and differentiation of, 555.
    media for, 73.
Collargol method for spirochætes, 128.
Collodion capsules, 163.
  membranes, 716.
Colonies, enumeration of, 91.
  rough, 39.
  smooth, 39.
Comma bacillus (*see* Cholera vibrio), 560.
Commensal, 172.
Common cold, 773.
Complement, 224, 227, 250.
  in anaphylaxis, 267.
  components of, 224.
  estimation of, 149.
  fixation of, 147.
  fixation and agglutination, 243.
    in glanders, 463.
    in gonorrhœa, 372.
    in tuberculosis, 437.
    in whooping-cough, 592.

Complement, guinea-pig, 150.
  as opsonin, 230.
Condyloma acuminatum, 788.
Conidia, 31, 859.
  of actinomyces, 468.
Conidiophore, 859.
Conjunctivitis, bacteria of, 314.
  gonorrhœal, 370.
  inclusion, 788.
Conorhinus megistus, 838.
Cooked meat medium, 59.
Copper sulphate medium, 73.
Cornet's forceps, 110.
Corrosive sublimate as fixative, 132.
Corynebacterium, 37, 396.
  acnes, 313.
    characters of, 314.
  diphtheriæ, 374.
  equi, 403.
  hofmanni, 401.
  ovis, 402.
  parvulum, 397.
  pyogenes, 403.
  renale, 403.
  ulcerans, 397.
  xerosis, 402.
Coryza, infectious, 773.
Cowpox, 729, 732.
Crescents in malaria, 791.
Cresol as antiseptic, 913.
Crithidial forms of trypanosomes, 827, 832.
Cryptococcus gilchristi, 880.
  linguæ pilosæ, 883.
Crystal violet, 113.
    as antiseptic, 911, 913.
    medium, 73.
Culture of anaerobic organisms, 92.
  media, agar, 60.
    beer wort agar, 76.
    bile-salt media, 73.
    blood, 68.
    of Boeck and Drbohlav, 817.
    boiled blood medium, 69.
    Bordet and Gengou's, 69.
    brilliant green enrichment, 74.
    broth, 56.
    carrot, 77.
    casein digest broth, 58.
    chocolate agar, 69.
    coagulated serum, 67.
    for coli-typhoid group, 73.
    cooked meat medium, 59.
    copper sulphate medium, 73.
    crystal violet medium, 73.
    Dieudonné medium, 70.
    digest bases, 56.
    for diphtheria bacilli, 75.
    egg media, 70.
    Endo's medium, 74.
    gelatin, 59.
Culture media, glucose agar, 61.
      broth, 56.
      gelatin, 60.
    glycerol agar, 61.
      broth, 56.
    Hartley' broth, 58.
    heart digest broth, 57.
    Hiss's serum water media, 79.
    Horgan and Marshall's medium, 75.
    hormone, 56.
    liver extract agar, 72.
    Löffler's blood serum, 68.
    Löwenstein-Jensen medium, 71.
    McLeod's medium, 75.
    milk, 72.
    Müller's tetrathionate medium, 75.
    oleate-blood-agar, 582.
    peptic blood digest, 583.
    peptone water, 58.
      agar, 61.
    potato, 71.
    preparation of, 55.
    reaction of, 61.
    Robertson's cooked meat medium, 59.
    Sabouraud's medium, 76.
    salt agar, 598.
    selective, 72.
    semi-solid agar, 61.
    serum, 67.
    Smith's method, 76.
    special, 67.
    thallium acetate medium, 73.
    tubing of, 82.
    use of, 82.
    Wilson and Blair's medium, 75.
Cultures, 83.
  bacterial, mounting of, 103.
  filtration of, 98.
  incubation of, 97.
  plate, 88.
  from single cells, 86.
Cutaneous reaction in diphtheria, 391.
    in scarlatina, 299.
    in syphilis, 676.
    in tuberculosis, 434.
Cutting of sections, 133.
Cyclogeny, 7.
Cystitis, 299, 317.
Cysts of endolimax nana, 816.
    entamœba coli, 814.
      histolytica, 813.
    iodamœba bütschlii, 816.
Cytase, 224.
Cytotoxic sera, 228.

Da Fano's minute bodies, 758.
Dansyz's bacillus, 538.
Dark-ground illumination, 107.
Dead cultures, immunity by, 205.
Decolorising agents, use of, 114.
Deep cultures, 83.
Deer-fly fever. 613.
Dehydration of sections, 112.
Delhi sore, 852.
Deneke's vibrio, 578.
Dengue, 784.
Dental abscess, 298.
Depression immunity, 205.
Dermacentor andersoni, 711.
  venustus, 711.
Dermacentroxenus rickettsi, 712.
Dermatitis, blastomycotic, 881.
Dermotropic viruses, 788.
Desensitisation, 263, 271.
Dettol as antiseptic, 913.
Dhobie itch, 870.
Dialister, 35.
  pneumosintes, 588.
Dick reaction, 299, 302.
  toxin, 300.
    preparation of, 302.
    purification of, 303.
    skin-test dose, 302.
Dientamoeba fragilis, 816.
Dieudonné medium, 70.
Digests for media, 56.
Diphtheria, 373.
  alum-toxoid, 393.
  antibodies in, 395.
  antitoxin, dosage, 395.
    therapeutic effects of, 394.
    use of, 390.
  bacillus, 373.
    antibodies, serological types, 395.
    association with other organisms, 382.
    avirulent, 398.
    biological types, significance of, 387.
    cultivation of, 377.
    distribution of, 379.
    effects of inoculation of, 382.
    fermentative properties, 378.
    gravis, 378, 388.
    hæmolysins of, 379.
    identification of, 396.
    inoculation with, 382.
    intermediate forms, 378.
    from the lower animals, 390.
    media for, 75.
    metachromatic granules of, 375.
    mitis, 378, 388.
    organisms allied to, 396.
    pathogenic effects of, 398.
Diphtheria bacillus, powers of resistance of, 379.
    staining of, 375, 376.
    stains for, 121.
    toxins of, 384.
    toxone of, 384, 385.
    virulence tests, 400.
      variations in, 386.
  of calves, 478.
  carriers, 389.
  diagnosis, 399.
    Folger method, 401.
  immunity against, 390.
  membrane, 380.
  methods of diagnosis, 399.
  Moloney test, 394.
  prophylactic immunisation against, 393.
  Schick test, 391.
  toxin, 385 *et seq*.
  toxin-antitoxin immunisation, 393.
  toxoid, 393.
  toxoid-antitoxin, 393.
    floccules, 394.
  toxoid immunisation, 393.
Diphtheroid bacilli, 396.
Diplobacillus liquefaciens, 316.
Diplococcus, 34.
  catarrhalis, 360.
  crassus, 360.
  intracellularis meningitidis, 350.
  lanceolatus, 326.
  liquefaciens of Petit, 316.
  mucosus, 360.
  pharyngis siccus, 360.
  pneumoniæ, 326.
Diplococci in throat, 359.
Disaccharides, 77.
Dissociation, microbic, 39.
Distemper of dogs, 721.
Disturbances of metabolism, 188.
Dorset's egg medium, 70.
Dreyer's standard agglutination, 140.
Drop method of making dilutions, 137.
Droplet infection, 887.
Drying *in vacuo*, 102.
Ducrey's bacillus, 321.
Duodenal ulcer, 313.
Durham's tube, 80.
Dust in relation to infection, 887.
Dysentery, amœbic, 809.
  amœbic, carriers of, 813.
    methods of examination in, 821.
  bacillary, 546.
    antiserum treatment of, 553.
    bacteriophage therapy, 553.
    immunisation against, 553.
    vaccination against, 553.

Dysentery bacilli, 546.
agglutination of, 550.
atypical, 551.
biological reactions, 548.
characters of, 547.
concomitants, 549.
Flexner, 548.
Flexner-Y sub-group, 548.
Gettings type, 552.
pathogenic properties of, 550.
relation to disease, 549.
Schmitz type, 552.
Shiga, 548.
Sonne type, 552.
toxins, of 550.
varieties of, 548.
in water, 894.
Y type of Hiss and Russell, 548.
lesions in, 818, 819.
Newcastle bacillus, 552.

Eberthella, 36.
typhi, 517.
Ectodermoses, 722.
Egg media, 70.
Ehrlich phenomenon, 219.
Ehrlich's antitoxic standard, 214.
rosindole reaction, 81.
side-chain theory, 246.
theory of toxic action, 198.
Eijkman's test, 509.
El Tor vibrio, 573.
Elementary bodies, 716, 720.
demonstration of, 725.
staining of, 725.
Embedding in paraffin, 133.
Emetine as chemotherapuetic agent, 918.
Encephalitis in animals, 762.
Australian, 761.
epidemic, 757.
experimental transmission of, 759.
Japanese, 761.
lethargica, 757.
post-vaccinal, 761.
St. Louis, 760.
spontaneous of rabbits, 762.
Encephalitozoön cuniculi, 762.
rabiei, 745.
Encephalo-myelitis, acute disseminated, 761.
Endocarditis, acute bacterial, 310.
bacteria in, 309.
experimental, 311.
gonorrhœal, 371
malignant, 310.
simple, 309.
subacute infective, 298, 309.
ulcerative, 285, 298, 310.
Endodermophyton, 870.
Endolimax nana, 816.
Endolimax, cysts of, 816.
Endo's medium, 74.
Endospores, 7.
Endotoxins, 191, 282.
of cholera vibrio, 569.
Entamœba buccalis, 822.
coli, 810.
characters of, 814.
cysts of, 814.
experimental infection with, 821.
dispar, 816.
gingivalis, 822.
histolytica, 809.
characters of, 810.
cultivation of, 816.
cysts of, 813.
distribution of, 818.
experimental inoculation with, 819.
minuta, 810.
nana, 814.
tetragena, 810.
Enteric fever, 534.
organisms described in, 534.
Enteritis, streptococcal, 298.
Enterococcus, 291.
Enumeration of colonies, 91.
Enzymes, 194.
Epidemic cerebro-spinal meningitis, 350.
encephalitis, 757.
experimental transmission of, 759.
and herpes, question of relationship, 764.
hiccup, 758.
influenza, 769.
poliomyelitis, 750.
cell inclusions, 754.
diagnosis of, 756.
experimental transmission, 751.
immunity, 755.
methods of investigation, 756.
mode of infection, 754.
pathology of, 753.
virus of, 752.
Epidermophyta, 869.
Epidermophyton cruris, 870.
rubrum, 870.
Epididymitis, 369.
Epitoxoid, 219.
Epizootic abortion, 615.
Eppinger's streptothrix, 477.
Ergophore group, 198.
Erysipelas, 304.
Erysipeloid, 482.
Erysipelothrix, 37, 482.
Erythema arthriticum epidemicum, 589.
Escherichia, 35, 507.

Escherichia, coli, 513.
communior, 513.
Escherich's bacillus, 513.
Esthiomène, 787.
Eubacteria, 2.
Eubacteriales, 34.
Eusol as antiseptic, 912.
Exaltation of virulence, 204.
Exotoxins, 190.
Eyre's method of standardising reaction, 66.

Fæcal streptococci in soil, 889.
Fæces, examination of, 168.
Farcin du bœuf, 478.
Farcy, 457.
Favides, 873.
Favus, 862.
parasites, cultures of, 864.
scutula, 868.
Female genital tract, normal bacterial flora, 923.
Fermentation, bacterial, 77.
Fermentation of sugars, 77.
tests, tubes for, 80.
Ferments, 196.
bacterial, 26.
Fever, 188.
Fibrinolysin, 296.
Fièvre boutonneuse, 712.
Film preparations, 109.
staining of, 113.
Filterability, factors determining, 715.
of organisms, 716.
of spirochætes, 717.
Filterable saprophytic organisms, 717.
virus diseases, of animals, 721.
immunity to, 720.
of man, 721.
viruses, 2, 715.
analogies with bacteria, 718.
biological relationships, 717.
classification of neurotropic viruses, 722.
cultivation of, 719.
in chorio - allanotic membrane, 727.
in minced chick embryo, 728.
withsurvivingtissuecells,728.
demonstration of, 723.
dermotropic, 722.
immunity reactions, 720.
methods of filtration, 723.
neurotropic, 722.
pantropic, 723.
preservation of, 728.
resistance to chemical agents, 719.
Filterable viruses, resistance to physical agents, 719.
sizes estimated by centrifuging, 717.
estimated by filtration, 716.
in tissue cultures, 728.
viscerotropic, 723.
Filter-passing viruses, 2, 715.
resistance to glycerol, 719.
Filters, bacterial, 98.
forms of, 98.
Filtration of cultures, 98.
sterilisation by, 54.
through graded collodion membranes, 716.
Finkler and Prior's vibrio, 577.
Fish, tuberculosis in, 427.
Fixation of complement, 147.
and hardening of tissues, 132.
of sections on slides, 134.
Fixing solutions, 111, 132.
Flagella, 10.
staining of, 124.
Flagellar agglutination, 141, 234.
Flagellata, 823.
Fletcher'smediumforleptospira,694.
Flexner-Y bacilli of dysentery, 548.
Flies, contamination of milk by, 904.
in relation to typhoid, etc., 529.
Flocculation, 140.
with diphtheria toxin, 217.
reaction in smallpox, 738.
in syphilis, 156, 677.
Fluorescein, 319.
Focal infection, 313.
Fontana's method for staining spirochætes, 128.
Food-poisoning, 282, 540.
bacteriological diagnosis, 540.
etiology, 540.
infection from animals, 540.
organisms associated with, 537, 539.
due to staphylococci, 282.
various types of Salmonella group associated with, 539.
Foot and mouth disease, 738.
virus of, 739.
Ford's modification of Mann's stain, 724.
Formaldehyde as antiseptic, 913.
Formalin solution as fixative, 132.
Forssman's antibody, 228.
Fortner's method for anaerobes, 96.
Foth's dry mallein, 463.
Fowl-pest, 721.
Fowlpox, 733.
Fowl typhoid, 545.
Frambœsia, 679.
Frei reaction, 787.
Friedländer's pneumobacillus, 348.

Fuchsin, basic, 113.
Fungi, asci, 859.
ascospores, 860.
basidia, 859.
biology of, 858.
conidia, 859.
conidiophore, 859.
diseases produced by, 862.
immunity and supersensitiveness to, 874.
imperfecti, 860.
methods of examination of, 864.
pathogenic, 858.
cultivation of, 864.
cultural characters of, 871.
methods of examination of, 864.
of skin, generalised lesions, 873.
supersensitiveness to, 873.
Furunculosis, 285.
Fusiform anaerobic bacilli, 662.
Fusiformis, 37.
fusiformis, 662.

Gaertner's bacillus, 538.
Gaffkia, 34.
tetragena, 318.
Gall-stones following typhoid fever, 524.
Gametocyte, 791, 795.
Garnet method for antiseptics, 909.
Gas formation, demonstration of, 80.
gangrene, 645.
metastatic, 651.
Gastric ulcer, 313.
Geissler's vacuum pump, 99.
Gelatin media, 59.
General paralysis, Tr. pallidum in, 671.
Wassermann reaction in, 677.
Gentian-violet, 113.
Giemsa's stain, 131.
for spirochætes, 129.
Glanders, 456.
diagnosis of, 464.
in horses, 456.
in man, 457.
serum reactions in, 463.
supersensitiveness in, 463.
bacillus of, 456.
agglutination of, 463.
characters of, 458.
cultivation of, 459.
experimental inoculation with, 460.
pathogenic action of, 461.
Globoid bodies, 752, 753.
Glossina morsitans, 834, 840.
palpalis, 832.
tachinoides, 833.
Glucose agar, 61.
broth, 56.
gelatin, 60.
Glycerol agar, 61.
broth, 56.
Gnezda's indole test, 82.
Goatpox, 732.
Gonidia, 7.
Gonococcus, 361, 363.
allergy, 371.
comparison with meningococcus 366.
cultivation of, 364.
distribution in tissues of, 368.
fermentation by, 367.
growth requirements, 366.
immunity, 371.
joint-affections, 370.
lesions caused by, 369.
oxidase reaction, 365.
pathogenic properties of, 367.
relations to disease, 367.
serological types of, 367.
serum diagnosis, 371.
toxin of, 368.
vaccines, 371.
variants, 365.
Gonorrhœa, 363.
complement fixation test in, 372.
methods of diagnosis, 372.
serum therapy, 372.
vaccines in, 371.
Gonorrhœal arthritis, 370.
conjunctivitis, 370.
endocarditis, 371.
septicæmia, 371.
Gradocol membranes, 716.
Grahamella, 857.
Gram's iodine, 117.
method, 116.
Jensen's modification of, 118.
Kirkpatrick's method for, 119.
Kopeloff and Beerman's modification of, 119.
modifications of, 117.
Weigert's modification of, 117.
Granulomata, infective, 187.
Group-agglutinins, 236.
measurement of, 142.
Guarnieri bodies, 735.
Gulland's method for fixing sections, 134.

Hæmagglutinins, 233.
Hæmatopinus spinulosus, 844.
Hæmolysin in malaria, 808.
normal, 227.
Hæmolysis by staphylococci, 281.
by streptococci, 287, 296.
Hæmolytic sera, action of, 225.

Hæmolytic streptococci, human lesions caused by, 297.
tests, methods of, 148.
Hæmophilic bacteria, 580.
resembling B. influenzæ, classification, 586.
Hæmophilus, 35.
conjunctivitidis, 314.
ducreyii, 321.
influenzæ, 579.
lacunatus, 315.
pertussis, 591.
Hæmosporidia, 789.
H-agglutination, 234.
Haffkine's vaccine for plague, 607.
Halogens as antiseptics, 912.
Hanging drop culture of moulds, 77.
preparations, 85, 107.
Haptens, 212, 238.
Haptophore group, 198.
Hartley's broth, 58.
Haverhill fever, 589.
Haverhillia multiformis, 589.
Heart digest broth, 57.
Heart-water, 703.
Helly's fluid, 133.
Hemispora stellata, 879.
Hemisporosis, 879.
Henry's method for anaerobes, 95.
Herd immunity, 257.
Herpes, cornealis, 763.
encephalitis in rabbits due to virus of, 763.
and epidemic encephalitis, question of relationship, 764.
experimental transmission of, 763.
febrilis, 763.
genitalis, 763.
virus, 763.
cell inclusions, 765.
immunity to, 764.
zoster, 766.
and varicella, question of relationship, 766.
Herpetic keratitis, 759, 763.
Heterogenetic antigen, 228.
Heterophile antibody, 228.
Higher bacteria, 3, 31.
Hippurate, hydrolysis of by streptococci, 292.
Hiss's method for staining capsules, 122.
serum water media, 79.
Hofmann's bacillus, 401.
Hog cholera, 721.
"Holder" method for milk, 906.
Horgan and Marshall's medium for B. diphtheriæ, 75.
Hormone media, 56.
Horsepox, 729, 732.
Hydrogen, supply of, 93.
Hydrogen-ion concentration, estimation of, 62.
Hydrogen peroxide as antiseptic, 912.
Hydrophobia, 739.
methods of diagnosis, 748.
pathology of, 741.
protective inoculation against, 745.
virus of, 742.
Hyphæ, 858.
Hyphomycetes of mycetoma, 480.
Hypochlorites as antiseptics, 912.
Hypodermic syringes, 160.

Idiosyncrasy, 269.
Immune-body, 211, 224.
action of, 225.
hæmolytic, estimation of, 148.
union of, 226.
Immune-opsonin, 229.
Immunisation, active, 301.
toxoid, 393.
Immunity, 200.
acquired, 200.
active, 201, 202.
methods of, 203.
artificial, 201.
methods of, 202.
by dead cultures, 205.
depression, 205.
by feeding, 208.
herd, 257.
infection, 250.
by living cultures, 204.
local, 209.
mass, 257.
natural, 200, 251.
non-sterilising, 177.
passive, 202.
methods of, 203.
by sensitised cultures, 206.
tissue, 250.
by toxins, 207.
unit, 216.
to virus disease, 720.
Impetigo, 298.
Inclusion bodies, 186.
staining of, 724.
of virus diseases, 719.
conjunctivitis, 788.
Incubation of cultures, 97.
India-ink method for spirochætes, 128.
Indicators, 79.
Indole production by bacteria, 80, 511.
Infant diarrhœa and milk, 904.
Infantile diarrhœa, bacilli of, 553.
Infection, 171.

Infection, conditions modifying, 178.
predisposing to, 172.
disturbances of metabolism in, 188.
droplet, 887.
immunity, 250.
mixed, 180.
and vitamin deficiency, 175.
Infectious coryza, 773.
Infective granules, 825.
Infectivity, 178.
Inflammatory conditions, 274.
methods of examination, 324.
Influenza, 579.
antiserum, 771.
epidemic, 769.
experimental transmission of, 770.
methods of examination in, 587.
in pigs, 771.
virus in, 770.
cultivation of, 772.
vaccine, 772.
bacillus, 579.
biological reactions, 583.
characters of, 580.
cultivation of, 580.
in diseases other than influenza, 585.
distribution of, 584.
experimental inoculation with, 586.
hæmolytic strains, 583.
in meningitis, 585.
methods of examination, 587.
serology of, 584.
V factor for growth, 582.
viability of, 584.
X factor for growth, 582.
Injection, methods of, 161.
Inoculating room, 87.
Inoculation of animals, 160.
Inspissation of serum, 67.
Intermittent sterilisation, 52.
Intracerebral injection, 163.
Intracutaneous injection, 162.
tuberculin test of Mantoux, 434.
Intraperitoneal injection, 161.
Intraspinal injection, 162.
Intravenous injection, 162.
Involution forms, 7.
Iodamœba bütschlii, 816.
cysts of, 816.
Iodine as antiseptic, 912.
Irido-cyclitis, 313.

Jahnel's method for staining spirochætes, 127.
Japanese encephalitis, 761.
Jaundice, infective, 692.
Jaundice "yellows," of dogs, 698.
Jenner's stain, 129.
Jennerian vaccination, 729.
Jensen's modification of Gram's method, 118.
Johne's bacillus, 446.
Johnin, 446.
Joints, gonococci in, 730.
Jungle yellow fever, 782.

Kala-azar, 844.
"Kataphylaxis," 174.
Keratitis, herpetic, 759, 763.
Kinetonucleus, 824, 845.
Kinetoplast, 824, 845.
Kipp's apparatus, 93.
Kirkpatrick's method for Gram's stain, 119.
method for staining capsules, 123.
method for staining flagella, 124.
Klebsiella, 35.
pneumoniæ, 348.
rhinoscleromatis, 349.
Klebs-Löffler bacillus (*see* Diphtheria bacillus), 373.
Koch's steam steriliser, 51.
Koch-Weeks bacillus, 314, 586.
Kopeloff and Beerman's modification of Gram's method, 119.
Koser's citrate utilisation reaction, 511.

Lactic acid bacilli, 900.
Lactobacillus, 35, 900.
Lamb dysentery, 653.
Laverania malariæ, 789, 802.
Leishman-Donovan bodies, 844.
Leishmania braziliensis, 853.
differentiation of, 853.
donovani, 844.
cultivation of, 847.
experimental infection with, 848.
infantum, 851.
tropica, 852.
Leishmaniasis, 844.
chemotherapy of, 918.
insect vectors, 849.
methods of examination, 850.
Leishman's stain, 130.
for trypanosomes, 824.
Lepra cells, 448.
Leprosy, 447.
bacillus of, 447.
cultivation of, 451.
distribution of, 450.
staining of, 449.
diagnosis of, 455.
etiology of, 453.
experimental transmission, 453.
mode of infection, 455.

Leprosy, pathological changes in, 447.
of rats, 453.
treatment with chaulmoogra oil, 455.
tuberculin reaction in, 454.
types of, 447.
Wassermann reaction in, 454.
Leptomonas, 848.
Leptospira, 37, 666.
adhesion reaction with, 696.
biflexa, 698.
canicola, 698.
cultivation of, 693.
grippo-typhosa, 699.
hebdomadis, 699.
icterohæmorrhagiæ, 692.
antibodies in, 695.
cultivation of, 693.
immunity reactions of, 695.
inoculation with, 696.
icteroides, 700.
identification of, 696.
pyrogenes, 700.
saprophytic, 698.
Leptothricia, 36.
Leptothrix, 482.
buccalis, 482.
Lesions produced by bacteria, 183.
toxins, 187.
Leucocidin, 193, 231, 281, 296.
Leucocytosis, 183.
Leukins, 254.
Levaditi's method for staining spirochætes, 126.
Lice in relapsing fever, 688.
trench fever, 713.
typhus fever, 705.
Light, effect of, 22.
Lignières and Spitz's actinobacillus, 477.
Liquor cresolis saponatus as antiseptic, 913.
Listerella, 35.
monocytogenes, 403.
Litmus, 79.
Liver abscess in dysentery, 818, 819.
extract agar, 72.
Local immunity, 209.
Löffler's blood serum, 68.
methylene-blue, 115.
Louping-ill, 762.
Löwenstein-Jensen medium, 71.
Lower bacteria, 2, 27.
Luetin, 676.
Lumbar puncture, 167.
Lustig's anti-plague serum, 609.
Lymphangitis, 298.
Lymphocytic chorio - meningitis, 762.
Lymphogranuloma inguinale, 787.
Lysol as antiseptic, 913.
Lysozyme, 255, 788.

MacConkey's bile-salt lactose agar, 73.
McFadyean's reaction for anthrax bacillus, 487.
McIntosh and Fildes' anærobic jar, 95.
McLeod's medium for B. diphtheriæ, 75.
Macrogamete, 798.
Macrogametocyte, 795.
Macronucleus, 824.
Mad itch, 748.
Madura disease, organisms of, 479.
Madurella, 481.
Mal de Caderas, 842.
Malaria, 251, 789.
benign tertian, 800.
blackwater fever in, 808.
chemotherapy of, 797, 808, 918.
control of, 804.
epidemiology of, 804.
experimental transmission of, 804.
hæmolysin in, 808.
immunity to, 807.
malignant, 802.
Maurer's dots, 802.
methods of examination, in 808.
of monkey, 805.
monocytosis of, 806.
mosquito transmission of, 804.
pathology of, 806.
plasmodium, 789.
asexual cycle in man, 790.
crescents, 791.
cultivation of, 803.
gametocyte, 791, 795.
macrogamete, 798.
macrogametocyte, 795.
merozoite, 791.
microgamete, 798.
microgametocyte, 795.
oökinete, 799.
ring-form, 793.
schizont, 791, 793.
sexual cycle in mosquito, 798.
sporoblast, 799.
sporocyst, 799.
sporozoite, 791, 799.
trophozoite, 791.
varieties of, 800.
zygote, 799.
quartan, 801.
quotidian, 801.
recurrences in, 797.
relapses in, 797.
relation of mosquito to, 798.

Malaria, Schüffner's dots, 800.
  sub-tertian, 802.
  therapy of general paralysis, 805.
  Ziemann's stippling, 801.
Malarial parasites, 789 *et seq.*
    cultivation of, 803.
    varieties of, 800.
Malassezia furfur, 884.
Malignant œdema, bacillus of, 654.
  pustule, 497.
Mallein, 462.
Malta fever, 615.
    agglutination in, 619.
    epidemiology of, 618.
    goats' milk as source of infection, 618.
  methods of diagnosis, 619.
  spread of disease, 618.
Mandler filter, 99.
Mann's method for fixing sections, 135.
  methyl blue eosin stain, 724.
Mantoux's tuberculin test, 434.
Margaropus annulatus, 855.
Mass immunity, 257.
Mastitis, bovine, 291, 292, 694.
Mastoiditis, 298.
Maurer's dots, 802.
Measles, 767.
  bacterial cause, question of, 768.
  serum prophylaxis in, 768.
  virus of, 767.
Mediterranean fever, 615.
Melioidosis, 464.
Meningitis, 298, 337, 499.
  due to B. influenzæ, 585.
  bacteria causing, 360.
  cerebro-spinal, 350.
  due to hæmophilic bacilli, 585.
  methods of examination, 362.
  precipitin reaction with cerebro-spinal fluid, 358.
  serum reactions, 358.
  tuberculous, 418.
Meningococcus, 350.
  agglutination of, 357.
  antisera for therapeutical purposes, 358.
  biochemical reactions of, 352.
  carriers of, 355.
  characters of, 350.
  cultivation of, 351.
  diplococci allied to, 359.
  distribution of, 353.
  experimental inoculation with, 353.
  fermentation by, 352.
  identification of, 356.
  infection, 354.
  preparation of agglutinating sera, 357.
Meningococcus, serological types, 356.
  toxins, 354.
  types of, 357.
  viability, 353.
Mercuric chloride as antiseptic, 909.
    as fixative, 132.
Mercurochrome as antiseptic, 912.
Mercury compounds as antispetics, 912.
Merozoite, 791.
Merthiolate as antiseptic, 913.
Metabolism, disturbances of in infections, 188.
Metachromatic granules, 11.
Metchnikoff's theory of phagocytosis, 231.
  vibrio, 574, 577.
Methylene-blue, 113.
    polychrome, 116.
    reduction test for milk, 907.
Methyl-red reaction of B. coli, 510.
    in water, 892.
Methyl-violet, 113.
Micro-ærophilic bacteria, 18.
Micrococcus, 27, 34.
  catarrhalis, 360.
  flavus, 359.
  rheumaticus, 306.
  tetragenus, 318.
Microgamete, 798.
Microgametocyte, 795.
Micro-manipulator, 87.
Micronucleus, 824.
Microscope, 106.
  plate culture, 91.
Microspora, 864.
Microsporides, 873.
Microsporum audouini, 865.
  equinum, 865.
  felineum, 865.
  furfur, 884.
  lanosum, 865.
  tardum, 865.
  umbonatum, 865.
  velveticum, 865.
Milk, B. abortus in, 622.
  B. coli in, 902, 907.
  B. melitensis in, 903.
  bacteriological standards of, 905.
  bacteriology of, 899.
  certified, 905.
  contamination by flies, 904.
  as a culture medium, 72.
  designations of, 905.
  graded, 905.
  methods of examination of, 907
  methylene-blue reduction test for, 907.
  pasteurisation of, 906.
  pathogenic organisms in, 903.

Milk, souring of, 900.
standards of, 905.
sterilisation of, 906.
tubercle bacilli in, 907.
Minimum lethal dose (M.L.D.), 214.
"Mist" bacillus, 428.
Mixed infection, 180.
Moeller's grass bacillus, 428.
Molluscum contagiosum, 788.
Moloney test, 394.
Monilia albicans, 875.
psilosis, 875.
tropicalis, 875.
Morax-Axenfeld bacillus, 315.
Morbilli, 767.
Mordants, use of, 114.
Morgan's No. 1 bacillus, 549, 554.
Mosaic disease of tobacco plant, 715, 718.
Mosquitoes and malaria, 804.
and yellow fever, 779.
Motility of bacteria, 10, 22.
examination for, 107.
Moulds, hanging drop culture of, 77.
media for, 76.
Mounting of bacterial cultures, 103.
Mouth, normal bacterial flora of, 921.
Much's method for tubercle bacillus, 405.
Mucor mucedo, 859, 861.
Muir's, Rd., method for staining capsules, 123.
Müller's bacillus, 315.
tetrathionate medium, 75.
Mumps, 768.
Mutation, 38.
Mycetoma, hyphomycetes of, 480.
Mycobacteriaceæ, 37.
Mycobacterium, 37, 408.
avium, 426.
berolinense, 428.
butyricum, 428.
lepræ, 447, 449.
paratuberculosis, 446.
phlœi, 428.
piscium, 427.
smegmatis, 429.
stercoris, 428.
tuberculosis, 405.
bovis, 422.
hominis, 407.
Mycosis of tongue, 482.
Myxobacteriales, 33.

Nagana, 823, 839.
Nasopharynx, examination of, 168.
normal bacterial flora of, 921.
Natural immunity, 200, 251.
supersensitiveness, 271.
Necrotoxin, 281.
"Negative phase," 215.
Negri bodies of rabies, 743.
Negro lethargy, 829.
Neisseria, 34.
catarrhalis, 360.
flava, 359.
flavescens, 360.
gonorrhœæ, 363.
meningitidis, 350.
sicca, 360.
Neisser's stain for B. diphtheriæ, 121.
use of, 376.
Neisser and Wechsberg, method of, 146.
Neisser-Wechsberg phenomenon, 225.
Neoplasms, 187.
Neurotropic virus, 782.
Neutral-red, 79.
action of B. coli on, 511.
Nicolle's medium for trypanosomes, 827.
Nitrates, formation of, 887.
reduction of by B. coli, 511.
Nitrifying bacteria, 24.
Nitrites, test for, 511.
Nocardia, 466.
Noguchi's medium for leptospira, 693.
Noma, 663.
Normal bacterial flora of alimentary tract, 922.
female genital tract, 923.
mouth, 921.
nasopharynx, 921.
paranasal sinuses, 921.
respiratory tract, 921.
skin, 920.
Novy and MacNeal's medium for trypanosomes, 827.
Nuclei of bacteria, 11.

O-agglutination, 234.
Obermeier's spirillum, 682.
Œdema, malignant, 654.
Ohara's disease, 613.
Oidium albicans, 875.
coccidioides, 882.
lactis, 862.
Oil immersion lens, use of, 106.
Oleate-blood-agar medium of Avery, 582.
O'Meara's modification of Voges and Proskauer's reaction, 510.
Oökinete, 799.
Oomycetes, 859.
Oospheres, 859.
Ophthalmia neonatorum, 370.

Ophthalmic tuberculin reaction, 434.
Opsonic action, 229.
index in pneumonia, 340.
methods, 143.
Opsonin, 229, 252.
Optoquine as chemotherapeatic agent, 919.
Ornithodorus moubata, 690.
Oroya fever, 856.
Osteomyelitis, bacteria of, 312.
experimental, 313.
suppurative, 285.
Otitis, 298.
media, 298, 337.
Oxidising agents as antiseptics, 911.
Ozæna bacillus, 321, 350.

Pancreatitis, 313.
Pappataci, 786.
Parabasal body, 846.
Paracholera, 573.
vibrios, 573.
Paracolon bacilli, 554, 549.
Paraffin, embedding and cutting in, 133.
Parafuchsin as chemotherapeutic agent, 916.
Paranasal sinuses, normal bacterial flora of, 921.
Parasites, 171.
Paratyphoid bacilli, 534.
agglutination of, 536.
fermentation reactions of, 535.
normal serum agglutination, 536.
preventive inoculation against, 536.
rough and smooth colonies of, 535.
Parotitis, infective, 768.
Paschen bodies, 734, 735.
Paschen's stain, 725.
Passive anaphylaxis, 263.
immunity, 202.
methods of, 203.
Pasteurella, 35.
group, 596, 611.
differentiation of members, 612.
serology and antigenic structure, 612.
pestis, 597.
Pasteurisation of milk, 906.
Pasteur's method of exaltation of virulence, 204.
vaccination against anthrax, 500.
hydrophobia, 745.
Pathogenic fungi, 858.
organisms, 171.
Pathogenic spirochætes, 666.
trypanosomes, 823.
Pathogenicity, 178.
Pediculus capitis, 705.
corporis, 705.
Penicillin, 588.
Penicillium, 862.
camemberti, 862.
expansum, 862.
Peptic blood digest medium of Fildes, 583.
Peptone water, 58.
agar, 61.
Periostitis, bacteria of, 312.
suppurative, 285, 298.
Peritonitis, 298, 317, 370.
pneumococcal, 338.
Peri-urethral abscess, 369.
Permanganate of potassium as antiseptic, 912.
Pestis major, 602.
minor, 602.
Petri and Rabinowitsch's butter bacillus, 428.
Petri's dishes, 88.
sand-filter method, 885.
Petroff's method for B. tuberculosis, 444.
Pfeiffer's influenza bacillus, 579.
phenomenon, 223.
reaction, 570.
Phagedænic lesions of the genitalia, 664.
Phagocytosis, 229, 252.
measurement of, 143.
theory of, 231.
Phenol as antiseptic, 913.
Phlebotomus argentipes, 849.
fever, 700, 786.
flies, 853.
intermedius, 853.
major, 852.
pappatasii, 786, 853.
perniciosus, 852.
sergenti, 853.
Photography by ultra-violet light, 717.
Phthioic acid, 411.
Phycomycetes, 858.
Physical characters of bacteria, 13.
Physiology of bacteria, 14.
Piedraia hortai, 874.
Pigeon-pox, 721.
Pigments of bacteria, 13.
Pipettes, measuring with, 136.
Piroplasma, 854.
bigeminum, 855.
parvum, 855.
Piroplasmosis, chemotherapy of, 918.
Pirquet's tuberculin test, 433.

Pitfield's method for staining flagella, 126.
Pityriasis versicolor, 884.
Plague, 596.
antisera, 609.
bacillus of, 596, 597.
agglutination of, 610.
bacteriophage, 610.
bubonic, 600.
cultivation of, 598.
distribution of, 600.
epizootics in various wild rodents, 606.
experimental inoculation with, 602.
fleas as vectors of, 605.
Haffkine's vaccine, 607.
immunity phenomenon in, 607.
infection in rats, recognition of, 611.
lesions in, 600.
methods of diagnosis, 610.
modes of infection in, 603.
pneumonia, 600.
infection in, 607.
preventive inoculation against, 607.
recognition of infection in rats, 611.
septicæmic, 601.
serum diagnosis, 610.
vaccine, 607.
Plakins, 254.
Plasmodium, 789.
falciparum, 789, 802.
knowlesi, 805.
malariæ, 789, 801.
ovale, 802.
vivax, 789, 800.
Plasmolysis, 13.
Plasmoquin as chemotherapeutic agent, 918.
Plate culture microscope, 91.
Plate cultures, 88.
Platinum wires, 84.
Pleuro-pneumonia, bovine, organisms of, 484.
Pneumobacillus, 348, 361.
of Friedländer, 348.
lesions caused by, 349.
serological types, 348.
Pneumococcal infections, 336.
serum therapy, 344.
Pneumococcus, 325, 360.
agglutination of, 343.
antisera, 339, 340, 344.
standardisation of, 346.
therapeutic mechanism of, 346.
biochemical reactions, 330.
characters of, 326.
cultivation of, 327.
Pneumococcus, experimental inoculation, 332.
hæmolysin, 328.
immunity to, 338.
infections, chemotherapy of, 919
lesions caused by, 335.
methods of examination for, 347.
mucosus, 327, 328, 331, 341.
prophylactic vaccination against, 347.
relations to streptococci, 330.
specific substance of, 342.
thrombolysin, 335.
transformation of types, 343.
types of, 340.
identification of, 343.
variants of, 330.
Pneumonia, 325.
prophylactic vaccination against, 347.
septic, 337.
treatment with antisera, 344.
types of, 336.
Poisons, animal, 196.
vegetable, 196.
Polar staining, 11.
Poliomyelitis, 257.
epidemic, 750.
pathology of, 753.
virus, 361.
Polychrome methylene-blue, 116.
Poradenitis, 787.
Post-diphtheritic paralysis, 374.
Post-mortems, bacteriological examination in, 169.
Post-operative wound infections, 297.
Post-vaccinal encephalitis, 761.
Potato media, 71.
Precipitation, mechanism of, 239.
Precipitin in pneumonia, 342.
Precipitins, 237.
and anaphylaxis, 265.
Preisz-Nocard bacillus, 402.
"Premunition," 177.
Preparation of vaccines, 158.
Proflavine as antiseptic, 913.
as chemotherapeutic agent, 918.
Prontosil as chemotherapeutic agent, 919.
Protective inoculation, 202 *et seq.*
Proteolytic group of anærobes, 647.
Proteosoma, 790.
Proteus, 36, 320.
capsulatus, 320.
mirabilis, 320.
vulgaris, 320.
X19, 709.
zenkeri, 320.
Protista, 1.
Prototoxoid, 219.

Protozoa, pathogenic, 789.
Pseudo-diphtheria bacillus, 401.
Pseudo-farcy, 402.
Pseudomonas, 35.
  æruginosa, 318.
Pseudo-rabies, 748.
  virus of, 748.
Pseudo-tuberculosis streptothricea, 478.
Psittacosis, 775.
  virus of, 776.
Ptomaine poisoning, 537.
Puerperal infection, 305.
  sepsis, 292, 298, 304.
  septicæmia, 298, 304.
Pugh's stain for B. diphtheriæ, 122.
" Puncture " cultures, 84.
Purple bacteria, 22.
Pus, preparations from, 110.
Pyæmia, 275, 285, 298.
Pyelitis, 317.
Pyocyanase, 319.
Pyocyanin, 319.
Pyogenic bacteria, 276.
    vaccine treatment, 323.
  cocci, 274.
    toxins of, 281, 295.
  infections, 309.
  organisms, mode of entrance and spread of, 276.
Pyorrhœa, 482.
  alveolaris, 298, 670.
Pyorubin, 319.
Pyosalpinx, 369.
Pyrogallic acid, use of, 95.

Quartan malaria, 801.
Quinine and blackwater fever, 808.
  as chemotherapeutic agent, 918.

Rabbits, encephalitis of, 762.
Rabies, 739.
  animal inoculation test, 748.
  antiserum, 747.
  diagnosis of, 748.
  Negri bodies in, 743.
  Pasteur's vaccine, preparation of, 745.
  pathology of, 741.
  prophylaxis by vaccination, 745.
  street virus, 745.
  transmitted by vampire bat, 741.
  types of, 740.
  vaccine, Hogyes' method, 746.
    neuroparalytic accidents, 747.
    Semple's method, 746.
  virus of, 742.
    fixe, 745.
Rabinowitsch's butter bacillus, 428.
Radiations, bactericidal effect of, 914.
Ramon's flocculation reaction, 217.
Rat trypanosomiasis, 843.
Rat-bite fever, 589, 700.
Rat viruses, 538.
Ratin, 539.
Rattus norvegicus (decumanus), 605.
  rattus, 605.
Rauschbrand bacillus, 658.
Ray fungus, 466.
Reaction of media, adjustment of, 61.
Receptors as antitoxin, 247.
Reducing agents as antiseptics, 911.
Reduction of nitrates by B. coli, 511.
Red-water fever, 855.
Refrigeration, 102.
Relapses in malaria, 797.
Relapsing fever, 682.
    immunity in, 686.
    louse transmission of, 688.
    mode of transmission, 689.
    serological reactions in, 686.
    spirochætes, 682.
      cultivation of, 683.
      experimental inoculation with, 685.
      immunological differentiation of, 691.
    tick transmission of, 689.
    varieties of, 688.
Relief staining for capsules, 124.
Resolution, microscopic, limit of, 716.
Respiratory tract, normal bacterial flora of, 921.
Reticulo-endothelial system, 175, 183, 244.
Rheumatism, acute, 305, 775.
  chronic, 313.
Rheumatoid arthritis, 313.
Rhinitis, 338.
Rhinoscleroma, bacillus of, 349.
Rhinosporidium, 879.
Rhipicephalus appendiculatus, 856
  sanguineus, 712.
  evertsii, 855.
Rhizoplast, 846.
Ricin, 196, 208, 218.
Rickettsia bodies, 703.
    staining of, 707.
  group, 703.
  infections, 703.
  melophagi, 704.
  mooseri, 710.
  prowazeki, 704, 706.
  quintana, 714.
  rickettsi, 712.
  rocha-limæ, 707.

Rideal and Walker's method for antiseptics, 909, 910.
Rieckenberg's adhesion phenomenon, 237, 837.
Rift Valley fever, 785.
Ring-forms of malarial parasites, 793.
Ringworm, 862
parasites, cultures of, 870.
small-spore, 864.
Robertson's cooked meat medium, 59.
Robin, 196.
Rocky Mountain fever, 711.
Romanowsky stains, 129.
Römer's test, 391.
Rose bengal, 116.
Rosindole reaction, Ehrlich's, 81.
Ross's "thick film process," 809.
Rous sarcoma virus, 721.
Rust-fungi, 860.

Sabouraud's medium, 76.
stain for trichophyta, 132.
Saccharolytic group of anaerobes, 647.
Saccharomycetaceæ, 862.
St. Louis encephalitis, 760.
Salivary gland virus of guinea-pigs, 724.
Salmonella, 36.
anatum, 544, 546.
choleræ-suis, 544.
var. Kunzendorf, 544.
classification of, 541.
enteritidis, 538.
gallinarum, 544, 545.
group, 537.
Aberdeen type, 540, 544.
antigenic and toxic fractions of, 540.
Bareilly type, 544, 545.
Dar-es-Salaam type, 544, 545.
Derby type, 540, 543, 544.
diphasic variation, 542.
Dublin variety of B. enteritidis, 540, 545.
Eastbourne type, 540, 544, 545.
Kunzendorf type, 543.
London type, 544, 546.
Newport type, 539, 544, 545.
Oranienburg type, 544, 545.
Poona type, 544, 546.
Potsdam type, 540, 544, 545.
Reading type, 543, 544.
Sendai type, 544, 545.
Senftenberg type, 540, 543.
var. Newcastle, 544.
serological classification of, 541.
serological identification of types, 546.
Salmonella group, Stanley type, 539, 543, 544.
Thompson type, 540, 544, 545.
var. Berlin, 544.
morbificans bovis, 545.
morgani, 554.
paratyphi, 534.
pullorum, 544, 545.
schottmülleri, 534.
typhi-murium, 544.
Salvarsan as chemotherapeutic agent, 917.
Sandfly fever, 786.
Sanocrysin as chemotherapeutic agent, 919.
Saprophytes, 171.
Sarcina, 30, 34.
Sarcoma of Rous, 721.
Satellitism of B. influenzæ, 582.
Scarification, 161.
Scarlatina, 299.
active immunisation, 301, 303.
antitoxic sera, 301.
antitoxin, standardisation, 304.
puerperal, 302.
serum therapy, 301, 303.
streptococci of, 299.
serological types in, 300.
surgical, 302.
Schick test, 391.
Schizogony, 790.
Schizomycetes, 2, 33.
Schizont, 791, 793.
Schizophyceæ, 4.
Schizophyta, 4.
Schizotrypanum, 839.
Schmitz's bacillus, 551.
Schüffner's dots, 800.
Schultz-Charlton reaction, 300.
Sclavo's anti-anthrax serum, 502.
Scutula, 868.
Sections, cutting of, 133.
fixation on slides, 134.
Gram's stain for, 119.
staining of, 114.
Sedimentation, 138.
Seitz filter, 99.
Selective media, 72.
Semi-solid agar, 61.
Sensitisation in anaphylaxis, 262.
Sensitised cultures, immunity by, 206.
vaccines, 206.
"Septic tank," 898.
Septicæmia, 181, 275, 285.
Serum, antianthrax, 502.
antibacterial, 222.
antidiphtheritic, 214.
antimeningococcal, 358.
antiplague, 609.
antipneumococcal, 339, 340, 344.

Serum, antistreptococcal, 309.
  antitetanus, 637.
  antitoxic, 213.
  antituberculous, 442.
  bactericidal action of, 145, 223, 253.
  bacteriolytic action of, 223.
  cytotoxic, 228.
  diagnosis of syphilis, 152.
  disease in man, 270.
  hæmolytic, 225.
  media, 67.
  method of obtaining, 165.
  precipitins, 238.
  steriliser, 54.
Seven-day fever, 699.
Sewage, activated-sludge process of purification, 899.
  bacteriology of, 897.
  bio-aeration method of purification, 899.
  purification, 897.
Sheeppox, 732.
Shiga's bacillus of dysentery, 548.
Shigella, 36.
  dysenteriæ, 548.
  paradysenteriæ, 548.
Shmamine's medium, 701.
Shwartzman phenomenon, 269.
Side-chain theory, Ehrlich's, 246.
Simon's method for opsonins, 145.
Single cell cultures, 86.
Skin, normal bacterial flora of, 920.
  pustules, 285.
Skin-test dose, 302.
" Slant " cultures, 84.
Sleeping sickness, 829.
    lesions in, 830.
    leucocytes in, 830.
" Slope " cultures, 83.
Smallpox, 729.
  antibodies in, 736.
  elementary bodies in, 734.
  flocculation test, 738.
  immunity reactions, 736.
  inclusion bodies 735.
  nature of virus, 729.
  Paul's test, 738.
  relationship to cowpox, 731.
  vaccination with cultivated virus, 734.
  virus, experimental inoculation, 731.
    nature of, 733.
Smegma bacillus, 429.
Smith, Th., phenomenon of, 262.
Smith-Noguchi medium, 672.
Smith's method for B. diphtheriæ, 76.
  tube, 80.
Smut-fungi, 860.
Snake venoms, 197.
    immunity against, 218.
Soaps as antiseptics, 913.
Sobernheim's anti-anthrax serum, 502.
Sodium hippurate, 292.
Sodoku, 700.
Soft sore, 321.
Soil bacteria, 889.
Soil, bacteriology of, 887.
  excretal bacteria in, 889.
  methods of examination of, 888.
Somatic agglutination, 141, 234.
Sonne type of dysentery bacilli, 552.
Sore-throat, streptococcal, 292, 298.
Specificity, 221.
  of antibodies, 210.
Spirillaceæ, 34.
Spirillum, 2, 30, 34.
  minus, 700.
Spirochæta berbera, 688.
  carteri, 688.
  crociduræ, 690.
  duttoni, 690.
  hispanica, 692.
  icterohæmorrhagiæ, 692.
  morsus muris, 701.
  novyi, 688, 692.
  obermeieri, 682.
  pallida, 667.
  persica, 692.
  recurrentis, 682, 918.
  turicatæ, 692.
  venezuelensis, 692.
Spirochætæ, 2, 31.
Spirochætal jaundice, 692.
    antibodies in, 696.
    antiserum treatment of, 698.
    diagnosis by inoculation of blood, 694.
    infection in, 698.
    preventive inoculation, 698.
    source and mode of infection, 697.
    spirochætes in urine, 695.
    therapeutic antisera in, 698.
Spirochætales, 37.
Spirochæte infections, chemotherapy of, 917.
Spirochætes, animal infections with, 682.
  pathogenic, 666.
  in relapsing fever, 682.
  staining of, 126.
  in Vincent's angina, 663.
Splenic fever (*see* Anthrax), 493.
Sporangium, 858.
Spore formation, 7.
Spores of actinomyces, 468.
  significance of, 8.
  staining of, 122.

Sporoblast, 799.
Sporocyst, 799.
Sporogony, 798.
Sporotrichosis, 876.
Sporotrichum beurmanni, 877.
Sporozoa, 789.
Sporozoite, 791, 799.
"Spreader," 320.
"Stab" cultures, 84.
Staining for acid-fast bacilli, 120.
  of bacteria, 112.
  of capsules, 122.
  of diphtheria bacillus, 121.
  of films, 113.
  of flagella, 124.
  principles of, 112.
  of sections, 114.
  solutions, 115.
  of spirochætes, 126.
  of spores, 122.
  for tubercle bacillus, 120.
Stains, Giemsa, 131.
  Jenner, 129.
  Leishman, 130.
  Romanowsky, 129.
  Wright, J. H., 131.
Stalactite growth, 599.
Standard agglutinable cultures, 140.
  of purity of water, 895.
Standardisation of antitoxin, 214, 216.
Staphylococci, 278, 361.
  active immunisation against, 301.
  biochemical reactions of, 280.
  carbohydrates, 285.
  classification of, 280.
  experimental inoculation, 284.
  human lesions caused by, 285.
  leucocidins, 281.
  necrotoxin, 281.
  toxins of, 281, 283.
  vaccines of, 285.
Staphylococcus, 34.
  anti-hæmolysin, 282.
  ascoformans, 280.
  cereus albus, 280.
    flavus, 280.
  citreus, 280.
  coagulase, 283.
  endotoxin, 282.
  epidermidis albus, 280.
  hæmolysins of, 281.
  immunity against, 285.
  pyogenes albus, 279.
    aureus, 278.
Stegomyia fasciata, 779.
Sterilisation by chemicals, 54.
  by dry heat, 50.
  by filtration, 54.
  by high pressure saturated steam, 52.
Sterilisation at low temperatures, 54.
  methods of, 49.
  of milk, 906.
  by moist heat, 51.
Sterility, maintenance of, 54.
Stomoxys calcitrans, 755
"Stormy clot" reaction, 649, 889.
Stormy fermentation of milk, 649.
Strangles' streptococcus equi, 294.
Streptobacillus moniliformis, 589, 702.
    pleuropneumonia - like symbiont, 590.
Streptococci, 286, 361.
  allergy to in acute rheumatism, 307.
  anærobic, 291, 292, 295, 305.
  antisera, 309.
  antitoxin, 309.
  bacteriæmia, 310.
  changes produced in blood media by, 289.
  classification of, 295.
  cultivation of, 286.
  endotoxin, 307.
    allergy to, 302.
  experimental inoculation, 296.
  fæcal, 291, 295.
    in soil, 889.
  fermentation reactions of, 288, 290.
  fibrinolysin, 296.
  in food poisoning, 308.
  hæmolysins of, 287, 289, 296.
  hæmolytic, human lesions caused by, 297.
  immunity to, 308.
  Lancefield's serological groups, 294.
  leucocidins, 296.
  morphology, 289.
  of mouth, 295.
  selective localisation of, 297.
  serological classification of, 293.
  toxins of, 295.
  vaginal, 305.
  variants, 288.
  viability, 288.
  in water, 893.
Streptococcus, 34.
  agalactiæ, 291, 292.
  anginosus, 290.
  Aronson's, 292.
  epidemicus, 286, 292.
  equi (Strangles'), 294.
  equinus, 290.,
  erysipelatis, 289, 304.
  fæcalis, 290.
  group, 288.
  hæmolyticus, 286.

Streptococcus, infections, chemotherapy of, 919.
lacticus, 292, 900.
lactis, 292.
longus, 286.
mesenteroides, 30.
mitis, 290.
mucosus, 293, 331.
pneumoniæ, 326.
pyogenes, 286, 290.
rheumaticus, 306.
salivarius, 290.
saprophyticus, 289.
scarlatinæ, 299.
antitoxic sera, 301.
identity of, 299.
serological classification of, 300.
toxins of, 299.
type α, 289.
type β, 289.
type γ, 289.
viridans, 289.
Streptothrices, 32.
in actinomycosis, 481.
pathogenic, other than actinomyces, 477.
Streptothrix, acid-fast, 478.
actinomyces, 466.
(Boström), 475.
characters of, 467.
(Israel and Wolff), 474.
Eppinger's, 477.
maduræ, 479.
" Stroke " cultures, 84.
Styryl quinoline dyes as chemotherapeutic agents, 916.
Subculture, 84.
Subcutaneous injection, 161.
Subdural inoculation, 162.
Substance sensibilisatrice, 211, 224, 226.
Sub-tertian malaria, 802.
Sugars, fermentation of, 77.
Sulphonamide as chemotherapeutic agent, 919.
Sulphur bacteria, 32.
Sulphuretted hydrogen production, 82.
Summer diarrhœa, 552.
Superinfection, 251.
Supersensitiveness, 260.
in glanders, 463.
natural, 271.
in tuberculosis, 432.
Suppuration, nature of, 274.
Suppurative conditions, 274.
Swamp fever, 699.
Swine erysipelas, 482.
influenza, 771.
Swinepox, 733.
Symplasm, 7.
Syngamy, 798.
Syntoxoid, 219.
Syphilis, 667.
chemotherapy of, 917.
flocculation reaction in, 156, 677.
immunity in, 675.
methods of examination, 678.
serum diagnosis of, 152.
transmission to animals, 673.
Wassermann reaction in, 677.
Syringes, 166.
hypodermic, 160.

Tellurite medium for B. diphtheriæ 75.
Tetanolysin, 634.
Tetanospasmin, 634.
Tetanus, 625.
antitoxin, use of, 638.
bacillus, 625.
agglutination of, 638.
characters of, 626.
cultures of, 628.
distribution of, 627.
isolation of, 628.
pathogenic action of, 630.
toxins of, 633.
dolorosus, 637.
experimental, 633.
idiopathic, 632.
immunity against, 637.
methods of examination in, 640.
preventive antitoxin treatment, 640.
toxin, action of, 635.
Tetrads, 30.
Tetrathionate medium of Müller, 75.
Texas fever, 855.
Thallium acetate medium, 73.
Theileria parva, 855.
Therapeutic substances regulations, 394, 441.
Thermophilic bacteria, 21.
Thiobacteria, 32.
Thionin, 113.
Throat swab, 168.
Thrush, 874.
Tick-borne fever, 762.
Tick fever, 690.
transmission of relapsing fever 689.
Timothy grass bacillus, 428.
Tinea, 862.
Tissue immunity, 250.
Tobacco plant, mosaic disease of, 715, 718.
Toluidine-blue, 113.
Tonsillitis, 298.
Torula, 862.

Torula histolytica, 884.
Toxic action, theory of, 198.
Toxins, animal, 196.
bacterial, 189.
diphtheria, 384.
estimation of, 214.
flocculation reaction with, 217.
of the gonococcus, 368.
immunity by, 207.
lesions caused by, 187.
minimum lethal dose of, 191.
nature of, 194.
preparation of, 214.
of staphylococci, 281.
of streptococci, 295.
of tetanus bacillus, 633.
variations in susceptibility to, 255.
vegetable, 196.
Toxoid immunisation, 393.
Toxoids, 191, 198, 219, 221.
Toxone, 384, 385.
Toxophore group, 198.
Trachoma, 788.
Trench fever, 713.
Treponema, 37, 666.
calligyrum, 670.
cuniculi, 678.
of framboesia, 679.
gracile, 669.
microdentium, 670.
minutum, 669.
mucosum, 670.
obermeieri, 682.
pallidum, 667.
cultivation of, 672.
dermotropic, 669.
distribution of, 670.
neurotropic, 669.
strains of, 668.
pertenue, 679.
refringens, 663, 669.
of yaws, 679.
Triatoma megista, 838.
Trichobacteria, 32.
Trichophyta, 865.
media for, 76.
Sabouraud's method for staining, 132.
Trichophytides, 873.
Trichophyton, 870.
acuminatum, 866.
crateriforme, 866.
ectothrix, 866.
endothrix, 866.
rosaceum, 866.
violaceum, 866.
Trichosporum, 874.
Trophonucleus, 824, 845.
Trophozoite, 791, 793.
Tropical ulcer, 852.
Trypaflavin as chemotherapeutic agent, 918.
Trypan-blue as chemotherapeutic agent, 916, 918.
Trypanocidal action of serum, 836.
Trypanosoma brucei, 836, 841.
congolense, 842.
cruzi, 838.
diagnosis of, 839.
equinum, 842.
equiperdum, 842.
evansi, 842.
gambiense, 830.
inoculation with, 831.
lewisi, 843.
melophagium, 843.
rhodesiense, 834, 839.
in animals, 839.
theileri, 843.
ugandense, 834.
vivax, 842.
Trypanosome fever, 833.
Trypanosomes, action of human serum on, 836.
cultivation of, 827.
development in fly, 832.
drug-fast strains, 916.
identification of, 828.
infectivity and pathogenicity of, 826.
metacyclic, 832.
methods of examination of, 837.
monomorphic, 825.
morphology and biology of, 823.
pathogenic, 823.
polymorphic, 825.
serum resistant, 837.
staining of, 824.
transmission of, 826.
Trypanosomiasis of animals, 839.
auto-agglutination of blood in, 838.
chemotherapy of, 916.
human, 829.
immunity in, 837, 917.
leucocytes in, 830.
methods of examination, 837.
Trypan-red as chemotherapeutic agent, 914, 916.
Tryparosan as chemotherapeutic agent, 916.
Tryparsamide as chemotherapeutic agent, 916.
Trypsin broth, 166.
Tsetse fly disease, 839.
Tsutsugamushi disease, 711.
Tubercle bacillus, 405.
aberrant forms, 409.
action of dead, 420.
action on the tissues, 415.

Tubercle bacillus antibodies, complement fixation test with, 437.
antiformin in examining for, 443.
avian type, 426.
BCG, 438.
dissociation of, 440.
immunisation with, 439.
bovine type, 421, 423.
and human types in animal disease, 426.
chemical composition of, 410.
cultivation of, 411, 444
cyanophile, 410.
dissociation, 413.
distribution of, 416.
experimental inoculation, 419
filter-passing forms, 410.
granular forms, 409.
human type, 407.
identification of types of, 421, 445.
inoculation test for, 443.
in milk, 907.
powers of resistance of, 414.
serology of, 437.
Spahlinger vaccine, 441.
specific reactions of, 433.
stain for, 120.
staining reactions of, 407.
Tuberculin Bazillenemulsion, 442
from cultures on synthetic media, 442.
in diagnosis, 433.
immunity phenomena in, 436.
Koch's new, standardisation, 442.
old, standardisation, 441.
-O, 441.
-R, 442.
reactions, 433.
test in cattle, 435.
varieties of, 441
Tuberculosis, agglutination in, 435.
allergy, 432.
in animals, 406.
antisera, 442.
avian, 426.
bovine, 421.
in cattle, 435.
chemotherapy of, 919.
complement fixation in, 437.
diagnostic methods, 442.
in fish, 427.
modes of infection in, 431.
specific reactions, 432.
supersensitiveness in, 432.
varieties of, 421.
Tularæmia, 613.
Typhoid bacillus, agglutination of, 520, 530.
Typhoid bacillus, antigens, 520.
biological reactions of, 519.
blood infection, 523.
characters of, 517
course of infection, 523.
cultivation of, 518.
H and O agglutination, 531.
in house flies, 529.
immunisation of animals against, 525.
invisible form of, 521.
isolation of, 555.
in milk, 529.
normal serum agglutination, 530.
relations to the disease, 526.
rough and smooth colonies of, 519.
in sewage, 529.
in soil, 890.
somatic and flagellar agglutinins, 531.
standard agglutinable suspensions, 531.
suppuration caused by, 523.
toxins, 525.
variants (R and S), 519.
Vi antigen, 521.
in water, 529.
carriers, 527.
fever, 506.
epidemiology of, 528.
gall-stones following, 524.
lesions in, 521.
pathogenic effects in animals of, 524.
pathology of, 521.
relationship of B. typhosus to, 526.
serum diagnosis of, 530.
suppurative lesions in, 523.
treatment with antisera, 533.
vaccination against, 532.
vaccine (T.A.B.), 536.
Typhoid - paratyphoid group in water, 894.
Typhus fever, 704.
agglutination in, 709.
heterologous agglutinins in, 719.
immunity phenomena, 708.
infective agent, 706.
murine, 710.
organisms isolated from, 708.
transmission of, 704.
varieties of, 710.
vector of infection, 705, 710.
Tyrode's solution, 728.

Ulcerative endocarditis, 285, 298, 310.

Ulcerative endocarditis, experimental, 311.
gingivitis, 663.
stomatitis, 663.
Ultramicroscopic viruses, 2. (*See* Filter - passing viruses, 715).
Ultra-violet light photography, 717
rays, effect of, 22.
Undulant fever, 615.
Undulating membrane, 824.
Unit, immunity, 216.
Unna's polychrome methylene-blue, 116.
Uredinaceæ, 860.
Urinary sepsis, 285.
Urine, examination of, 169.
tubercle bacilli in, 418
typhoid bacilli in, 559.
Urobacillus septicus, 320.
Ustilaginaceæ, 860.

Vaccination, Jennerian, 729.
against smallpox, 729.
Vaccine, Calmette's BCG, 438
lymph for smallpox immunisation, 730.
Vaccines, 203.
in coryza, 774.
in gonorrhœa, 371.
preparation of, 158.
sensitised, 206.
staphylococcal, 285.
treatment by, 207, 323.
Vaccinia, 729.
antibodies in, 730.
elementary bodies, 734.
immunity reactions, 736.
inclusion bodies, 735.
virus, cultivation of, 734.
experimental inoculation, 731.
immunity reactions, 737.
nature of, 733.
resistance to various agents, 735.
size of, 734.
Variants, rough, 39.
smooth, 39.
Variation among bacteria, 38.
Varicella, 766.
and herpes zoster, question of relationship, 766.
Variola, 201, 729.
(*See* Smallpox), 729.
Vegetable poisons, 196.
immunity against, 218.
Venins, 197.
Verruga peruviana, 857.
Vi agglutination, 142.
antibodies, 234.
Vi agglutination antigens, 234.
Vibrio, 2, 30, 34.
choleræ (*see* Cholera vibrio), 560.
isolation from water, 896.
comma, 560
of Deneke, 578.
El Tor, 573
of Finkler and Prior, 577.
fœtus, 578.
metchnikovi, 574, 577.
paracholeræ, 573.
proteus, 574.
Vibrion septique, 654.
cultures of, 655.
experimental inoculation with, 657.
immunity to, 658.
toxin, 658.
Vibrios, "cholera-like," 574.
phosphorescent, 574, 578.
Vincent's angina, 663.
Virulence, 178, 213, 235.
attenuation of, 203.
exaltation of, 204.
Virus diseases, immunity to, 720.
III of rabbits, 724.
Viruses, filterable, 715.
ultramicroscopic, 715.
Viscerotome, 779.
Visibility, microscopic, limit of, 716.
Vitamins in bacteria, 13
deficiency and infection, 175.
Voges and Proskauer's reaction, 510
for B. coli in water, 892.
Volutin 11, 12.

Walbum's medium for production of staphylococcal toxins, 283.
metallic salt therapy, 919.
Warts, 787.
Wassermann reaction, 152, 677.
antigen for, 154.
in bezel, 681.
in frambœsia, 681.
in general paralysis, 677.
in leprosy, 454.
methods, 154.
in yaws, 681.
test of cerebro-spinal fluid, 155.
Water, B. coli test for purity of, 891, 895.
B. dysenteriæ in, 894.
B. welchii, detection of, 892.
bacterial content of, 891, 893.
bacteriology of, 890.
cholera vibrio in, 896.
collection of samples, 890.
contamination of, by sewage, 894.

Water, filtration of, 894.
methods of examination of, 890.
pathogenic bacteria in, 894.
standard of purity of, 895.
streptococci, detection of, 892.
typhoid-paratyphoid group in, 896.
vibrio choleræ in, 896.
Weigert's modification of Gram's method, 117.
Weil-Felix reaction, 321, 709.
Weil's disease, 692.
Wenyon's medium for leptospira, 694.
Whole-blood, bactericidal action of, 147.
Whooping-cough, 590.
bacillus, characters of, 591.
diagnosis, 595.
methods of examination in, 595.
question of a filterable virus in, 594.
vaccination against, 594.
Widal reaction, 530, 536.
in vaccinated persons, 536.
Wilson and Blair's medium, 75.
Woolsorter's disease, 498.
Wound infections, chemotherapy of, 918.
Wounds, suppurating, 285.
Wright's, A. E., method for bactericidal action, 146.
method for opsonins, 143.
J. H., stain, 131.
Wright's vaccine methods, 158.

X-disease, 761.
Xenopsylla cheopis, 605, 710.
Xerosis bacillus, 316, 402.
Xylol as clearing agent, 112.

Yaws, 679.
Yeasts, 862.
Yellow fever, 777.
causation of, 779.
cell inclusions in, 781.
experimental transmission of, 779.
filterable virus of, 780.
histological changes in liver, 778.
immunity, 783.
jungle, 782.
leptospira, 781
preventive inoculation, 783.
virus of, 780.
neurotropic, 782.
viscerotropic, 782.
"Yellows" of dogs, 698.
Yersin's anti-plague serum, 609.

Zenker's solution, 133.
Zenodiagnosis, 839.
Ziehl-Neelsen carbol-fuchsin, 116.
Zone phenomenon, 240.
Zoogloea, 4.
Zygomycetes, 859.
Zygospore, 859.
Zygote, 799.